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Science



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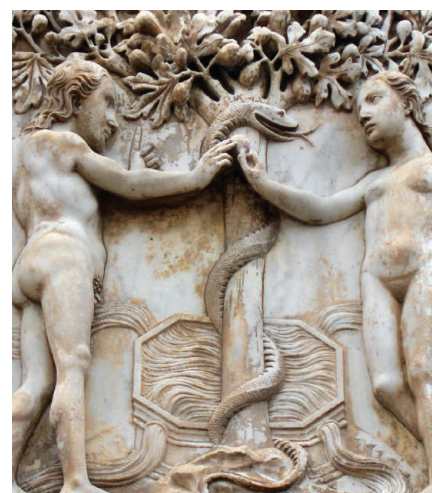
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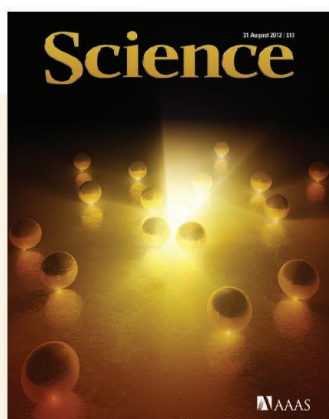
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Image: Sebastian Nicosia and Cristian Ciraci

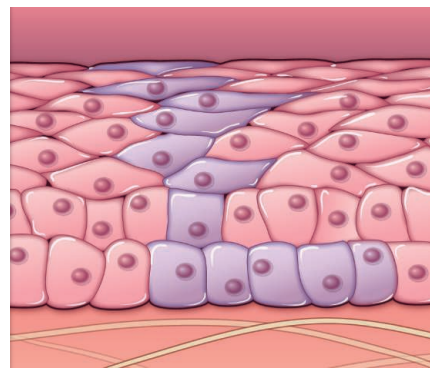
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10.1126/science.1228380

A High-Coverage Genome Sequence from an Archaic Denisovan Individual

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Processing and Subcellular Trafficking of ER-Tethered EIN2 Control Response to Ethylene Gas

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10.1126/science.1225974

Disulfide Rearrangement Triggered by Translocon Assembly Controls Lipopolysaccharide Export

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10.1126/science.1227215

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Comment on "Intensifying Weathering and Land Use in Iron Age Central Africa"

K. Neumann et al.

Full text at www.sciencemag.org/cgi/content/full/337/6098/1040-c

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J. Maley et al.

Full text at www.sciencemag.org/cgi/content/full/337/6098/1040-d

Response to Comments on "Intensifying Weathering and Land Use in Iron Age Central Africa"

G. Bayon et al.

Full text at www.sciencemag.org/cgi/content/full/337/6098/1040-e

SCIENCENOW

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Highlights From Our Daily News Coverage

What Time Is It on Your Circadian Clock?

Researchers find an easier way to determine our internal body time.
<http://scim.ag/Circadian-Clock>

New Computer Memory Material Goes Easy on the Juice

Organic compounds could help retain data without a continuous trickle of electricity.
<http://scim.ag/Organic-Compounds>

Genome Sequencing Clears Up a Cancer Medical Mystery

A mutation in a patient's tumor explains her rare response to a new drug.
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SCIENCE SIGNALING

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The Signal Transduction Knowledge Environment

28 August issue: <http://scim.ag/ss082812>

RESEARCH ARTICLE: A VASP-Rac-Soluble Guanylyl Cyclase Pathway Controls cGMP Production in Adipocytes

K. Jennissen et al.

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By suppressing cAMP production in the striatum, the RGS9-2/Gβ₃ complex could affect the development of opioid addiction.

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The role of β cell taste receptors in the control of insulin secretion is unclear.

PODCAST

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A protease can help protect against neurodegeneration by preventing inflammation in microglia.

SCIENCE TRANSLATIONAL MEDICINE

www.sciencetranslationalmedicine.org

Integrating Medicine and Science

29 August issue: <http://scim.ag/stm082912>

RESEARCH ARTICLE: Clonal Evolution of Preleukemic Hematopoietic Stem Cells Precedes Human Acute Myeloid Leukemia

M. Jan et al.

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RESEARCH ARTICLE: A Dense Poly(Ethylene Glycol) Coating Improves Penetration of Large Polymeric Nanoparticles Within Brain Tissue

E. A. Nance et al.

Nanoparticles densely coated with poly(ethylene glycol) rapidly penetrate within mouse, rat, and human brain parenchyma.

RESEARCH ARTICLE: The Stoichiometric Production of IL-2 and IFN-γ mRNA Defines Memory T Cells That Can Self-Renew After Adoptive Transfer in Humans

A. Wang et al.

Cytokines can identify memory T cells used for cancer immunotherapy.

FOCUS: FDA Oversight of Cell Therapy Clinical Trials

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FOCUS: Designing a Public Square for Research Computing

D. R. Masys et al.

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SCIENCE CAREERS

www.sciencereers.org/career_magazine

Helping Paralympians Go for Gold

E. Pain

Sports biomechanics researcher Barry Mason works on improving wheelchair design for basketball and rugby athletes.

<http://scim.ag/Paralympians>

Spotlight on Diversity

M. Price

Filmmaker and physicist Aziza Baccouche, who is blind, showcases the challenges and successes of diverse scientists in a new documentary series.

<http://scim.ag/DiversityDocumentary>

SCIENCEPODCAST

www.sciencemag.org/multimedia/podcast

On the 31 August Science Podcast: a reservoir of antibiotic resistance, forests that control the rain, the latest in Denisovan DNA, and more.

SCIENCEINSIDER

news.sciencemag.org/scienceinsider

Science Policy News and Analysis

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ADVANCING SCIENCE. SERVING SOCIETY

Mitochondrial Dynamics

Mitochondria—the powerhouses of the cell—are autonomous organelles with their own genomes. Within cells, mitochondria are remarkably dynamic, continually moving around the cytoplasm and undergoing fusion and fission reactions. **Youle and van der Bliek** (p. 1062) review the importance of mitochondrial fusion and fission in cellular responses to stress, interference with which are likely to play an important role in a variety of diseases including Parkinson's disease. In their Perspective, **Hoppins and Nunnari** explain that the endoplasmic reticulum is an active participant in mitochondrial division and discuss how mitochondrial dynamics and cell death are linked.

Dark Forcing

Soot, or black carbon, is a ubiquitous atmospheric pollutant whose warming effect might be second only to carbon dioxide. When black carbon is emitted, it combines with other aerosols to form heterogeneous mixtures. Models have predicted that internal mixing of black carbon with other materials can double the amount of radiation absorbed. **Cappa et al.** (p. 1078) report that in situ measurements of the enhancement of radiation absorption by these mixed black carbon-containing particles in the atmosphere show a much smaller effect. Thus, many climate models may be overestimating the amount of warming caused by black carbon emissions.

Vibrating in a Crowd

High-vacuum molecular beam studies can probe the roles of specific vibrations and rotations on molecular reactivity with remarkably fine resolution. **Glowacki et al.** (p. 1066; see the Perspective by **Tyndall**) now show, through a combination of spectroscopy and theoretical modeling, that oxidation of acetylene under effectively atmospheric conditions proceeds in part through vibrationally excited intermediates prior to collisional randomization.

Pretend Wires

Cold atomic gases have been successfully used to simulate solid-state phenomena such as quantum criticality. However, simulating mesoscopic electronic transport like that realized in

quantum wires is challenging. **Brantut et al.** (p. 1069, published online 2 August) connected two reservoirs of fermionic ^6Li atoms (simulating electrons) with a narrow channel (simulating a wire), created a nonequilibrium situation by applying a magnetic field gradient, and observed the flow through the channel. When the mean-free path of the atoms exceeded the length of the channel, the atomic density in the channel was constant in the central region and only changed at the ends, indicating the presence of contact resistance. The opposite diffusive regime created by imposing a disordered laser potential produced a uniformly varying density inside the channel.

Boundaries on Plasmonic Excitations

The localization of optical fields within a metal nanostructure can achieve strengths that are orders of magnitude greater than that of the incident field. This focusing and enhancement of the optical field maybe useful in sensing, nonlinear optics, and optical scattering applications. In probing the properties of metallic nanoparticles, **Ciraci et al.** (p. 1072; see the cover) show that the enhancement is limited by the electronic response of the metal, which has implications for the ultimate performance of nanophotonic systems.

Salty Origins of Fresh Water

Cloud droplets above the Amazonian rainforest form mostly around organic aerosols, but the source of the aerosols has been a mystery. **Pöhlker et al.** (p. 1075) report that particles rich in potassium salts emitted by Amazonian vegetation can act as the seeds for the growth of organic aerosol particles that function as condensation nuclei for water droplets. These specks of biogenic salts provide a surface for the condensation of low- or semi-volatile organic compounds formed by the atmospheric oxidation of isoprene and terpenes, molecules produced in great abundance by many kinds of Amazonian plants.

Curls Beget More Curls

Cucumber tendrils reach up to find an attachment, and then coil to shorten and drag the plant up toward the sunlight. **Gerbode et al.** (p. 1087) analyzed the biomechanics of cucumber tendril coiling. The process depends



Natural Selection at Work

Catching the evolution of a novel function and determining its selective parameters in nature remains an extremely difficult task. **Prasad et al.** (p. 1081) have undertaken this quest documenting the molecular basis of a natural allelic polymorphism and its effects on herbivory and survival in the *Arabidopsis* relative, *Boechera stricta*, living in the Rocky Mountains.

on a thin layer of cells within the tendril that becomes lignified during the coiling process. A construct of pre-strained silicon sheets, fabric ribbon, and copper wire reproduced the coiling functions in abiotic materials. Physical and mathematical models explained the peculiar response by which the cucumber tendril initially overwinds when pulled further.

Skin Specifics

Much of the recent attention paid to the trillions of bacteria that colonize our bodies has been given to the bacteria that reside in the gut. **Naik et al.** (p. 1115, published online 26 July) report that colonization of the skin with commensal bacteria is important for tuning effector T cell responses in the skin and for protective immunity against cutaneous infection with the parasite *Leishmania major* in mice. In contrast, selective depletion of the gut microbiota, which plays an important role in modulating immune responses in the gut, had no impact on T cell responses in the skin.

A Fungal Culprit to Carbon Loss

In some ecosystems, such as in the layer of soil containing plant roots, fungi, and bacteria, increased levels of CO₂ should stimulate more efficient aboveground photosynthesis, which in turn should promote increased sequestration of organic carbon in soil through the protective action of arbuscular mycorrhizal fungi. However, in a series of field and microcosm experiments performed under elevated levels of CO₂ thought to be consistent with future emissions scenarios, **Cheng *et al.*** (p. 1084; see the Perspective by **Kowalchuk**) observed that these fungi actually promote degradation of soil organic carbon, releasing more CO₂ in the process.

Epithelial Defense Force

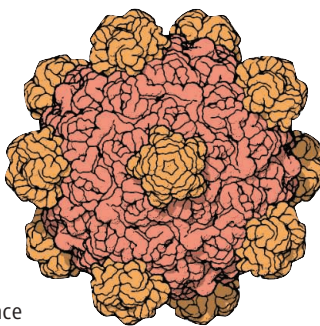
The nature of the cells that maintain and heal the epithelium lining the esophagus has been controversial. **Doupé *et al.*** (p. 1091, published online 19 July; see the Perspective by **Kushner**) show that, unlike many other tissues, mouse esophagus is devoid of slow cycling stem cells. Instead, the epithelium is maintained and repaired by a single population of proliferating cells that can switch rapidly from homeostatic behavior into “repair mode” in the vicinity of a wound.

Modulating the Clock

Because of the close association of the circadian clock with a wide range of physiological processes, identification of clock-modulating small molecules may prove useful for the treatment of circadian-related disorders, which include circadian sleep disorders, cardiovascular disease, cancer, and metabolic disease. **Hirota *et al.*** (p. 1094, published online 12 July) screened for chemical compounds that affected the period of the circadian clock in a human osteosarcoma cell line. A carbazole derivative named KL001 appeared to act by inhibiting proteolytic degradation of the cryptochrome proteins, which in turn caused a lengthening of the circadian period. KL001 also inhibited glucagon-induced gluconeogenesis in primary cultures of mouse hepatocytes.

Keeping DNA Flexible

The elastic behavior of DNA is important to biological processes that involve DNA bending and looping. However, there has been considerable debate over the flexibility of DNA at lengths below the persistence length (around 150 base pairs). A widely used approximation, the wormlike chain model, predicts stiff short DNA. **Vafabakhsh and Ha** (p. 1097; see the Perspective by **Nelson**) directly monitored cyclization of single molecules of DNA, by using a fluorescence assay, and found significant looping, with the looping rate having only weak length dependence between 67 and 105 bp, which is inconsistent with the wormlike chain model. Instead, DNA binding proteins may stabilize transiently bent or looped DNA conformations.



Good Enough Can Be Good Enough

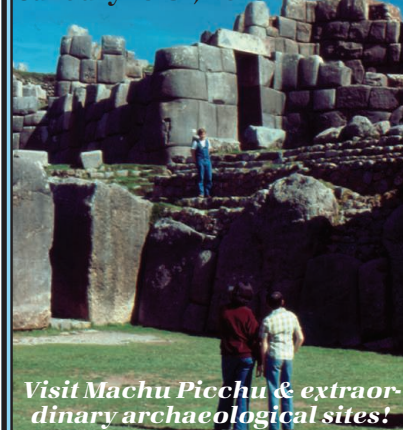
To begin to understand why some enzymes are promiscuous and have many substrates, whereas others are highly specific, and why some have high activity, whereas others appear not to be optimized, **Nam *et al.*** (p. 1101) analyzed metabolic networks in bacteria. Specialist enzymes are essential for life, catalyze a high flux of enzymatic activity, and are more highly regulated. However, not all enzymes appear to be on a track of gradual improvement of specificity and efficiency. Generalist enzymes seem to well serve their own purposes, and their optimization may not justify the evolutionary cost.

From Farm to Clinic?

Soil organisms have long been assumed to be an important source of antibiotic resistance genes, in part because of antibiotic-treated livestock and in part because of the natural ecology of antibiotic production in the soil. **Forsberg *et al.*** (p. 1107) developed a metagenomic protocol to assemble short-read sequence data after antibiotic selection experiments, using 12 different drugs in all antibiotic classes, and compared antibiotic resistance gene sequences between soil bacteria and clinically occurring pathogens. Sixteen sequences, representing seven gene products, were discovered in farmland soil bacteria within long stretches of perfect nucleotide identity with pathogenic proteobacteria.

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Ending Honorary Authorship

CREDIT FOR SCIENTIFIC RESEARCH CONTRIBUTIONS MUST BE CLEARLY AND APPROPRIATELY ASSIGNED at the time of publication. This task has become increasingly complicated because of the number of different laboratories and coauthors involved in many studies. The good news is that academic institutions, funders, and publishers are exploring new ways to clarify attribution,* and many publishers now require disclosure of specific contributions for scientific authorship. As part of this effort, it is critical that the problem of honorary authorship be effectively addressed. According to a recent report, honorary authors were attached to 25% of research reports, 15% of review articles, and 11% of editorials published in six major medical journals in 2008.† It is time to end this practice.

A true author is someone who has made substantive intellectual contributions to a study and is responsible for a component of the work. Honorary authorship violates this central principle. Why then is it so frequent? In some cases, honorary authorship amounts to “coercive authorship,” in which a senior person informs a junior colleague that the senior person must be listed as an author, even though she/he did not contribute substantially—or at all—to the work. In other cases, the principal investigator may add the name of a prominent scientist in the field as a guest author in an attempt to boost the paper’s chance of publication. Both types of behavior have fraudulent aspects, distorting the ethical culture that is central to a healthy academic environment.

To discourage honorary authorship and ensure appropriate accountability for published results, many journals have updated their policies on authorship. For some (including *Science*), all authors must formally agree to be listed as authors, specify their contributions to the manuscript, and certify that they approve of its content and submission to the journal. But scientific journals could go even further by adding a statement on authorship forms that reminds authors of their accountability in the event of challenges to the veracity or integrity of the work, such as “By signing this statement, I acknowledge that I take credit for the content of the published work. I also acknowledge that I will take responsibility for the work if questions arise in the future as to its authenticity and credibility.” Such a statement would serve as a firm reminder that being inappropriately listed as an author has negative consequences if the results are challenged or retracted.

Research institutions should develop and promulgate clear statements in their research policies about the importance of upholding ethical standards of authorship. For example, Washington University in St. Louis‡ defines both guest and gift authorship as research misconduct, whereby “guest (honorary, courtesy, or prestige) authorship is defined as granting authorship out of appreciation or respect for an individual, or in the belief that expert standing of the guest will increase the likelihood of publication, credibility, or status of the work” and “gift authorship is credit, offered from a sense of obligation, tribute, or dependence, within the context of an anticipated benefit, to an individual who has not contributed to the work.” Each institution should also specify to whom concerns should be directed, without fear of retribution, when an author feels coerced to include an inappropriate author.

It is incumbent on more-senior coauthors to assist in educating their colleagues about the proper standards for authorship. But all scientists should take a stand against coercive authorship and refuse to comply with such behavior. In this way, senior faculty and mentors will serve as role models of best practices, reinforcing for more-junior investigators the importance of ensuring appropriate authorship. Honorary authorship must no longer be tolerated. Concerted efforts by institutions, authors, and journals are needed to put an end to this fraudulent and unethical practice.

— Philip Greenland and Phil B. Fontanarosa

10.1126/science.1224988

*http://projects.iq.harvard.edu/attribution_workshop. †J. S. Wislar *et al.*, *Br. Med. J.* **343**, d6128 (2011).

‡<http://wustl.edu/policies/authorship.html>.



ECOLOGY

Unlocking the Secrets of a Lost World

The ancient sandstone table mountains, or tepuis, of the tropical South American Guayana Shield are legendary “lost worlds” renowned for their inaccessibility, mystery, and isolation. Rising hundreds of meters vertically from the surrounding savannas and forests, the summits of individual tepuis are known to harbor high percentages of endemic species of plants and animals that have evolved in isolation over millions of years. Or do they? Kok *et al.* helicoptered onto the summits of 17 tepuis to take tissue samples from amphibian species for genetic analysis. Phylogenetic analysis of mitochondrial gene fragments indicated surprisingly close affinities between many of the taxa on separate peaks, indicating that the barriers to gene flow may have been less complete than hitherto thought. The genetic data suggest that dispersal between summits may have been taking place through the Pleistocene and into the Holocene, so that substantial elements of the fauna may be less than 1 million years old—far less than the forbidding nature of the tepuis would seem to predict. — AMS

Curr. Biol. **22**, R589 (2012).

CHEMISTRY

Sugar Placement

Chemical bonds vibrate at frequencies that depend on the masses of the linked atoms. Because bond scission and formation are essentially extreme sorts of vibration, their rates also vary when the atom masses change, giving rise to kinetic isotope effects that offer insight into the order and extent of bond rearrangements underlying a reaction. The easiest, and thus most common, effects to study involve deuterium/hydrogen substitutions, given the factor of 2 mass difference. The 13/12 mass ratio of stable carbon isotopes induces a smaller rate distinction that is nonetheless discernible—even at the low natural abundance of ^{13}C —using current nuclear magnetic resonance (NMR) technology. Huang *et*

al. applied this technique to elucidate the precise mechanistic details of substitution reactions at the anomeric carbon of simple sugars, which bear on the selectivity attainable in the generating particular oligosaccharides for targeted biological studies. Specifically, they examined rate distinctions for ^{12}C versus ^{13}C centers in the displacement of trifluoromethanesulfonate by isopropanol to form the α and β anomers of mannopyranoside and glucopyranoside. For three of the four reactions, comparison of the NMR data to theoretical simulations supported a loosely associative mechanism, with the bond-cleaving and α -forming events perhaps just shy of simultaneous. The β -mannopyranoside was unusual in appearing to form through initial bond scission before isopropanol binding. — JSY

Nat. Chem. **4**, 663 (2012).

EDUCATION

Making Use of Misconceptions

Ironically, educators themselves hold misconceptions on how best to deal with their students' preexisting ideas. Instead of categorizing misconceptions as mistakes needing to be managed, is it possible to use them as a resource for learning? Larkin surveyed 14 preservice science teachers in different teacher preparation programs and found that their views on student misconceptions fell into five general categories: evidence of content coverage, obstacles to understanding, tools to encourage thinking, elements of a positive classroom environment, and the raw material of learning. Over the course of learning to teach, preservice teachers adjusted their view of student misconceptions, and most grew to recognize the teaching potential of misconceptions. These results suggest that teacher educators should encourage preservice teachers to incorporate misconceptions into their teaching as learning platforms to build on, instead of obstacles to learning. — MM

Sci. Educ. **96**, 927 (2012).

PLANT SCIENCES

How Bananas Weather a Drought

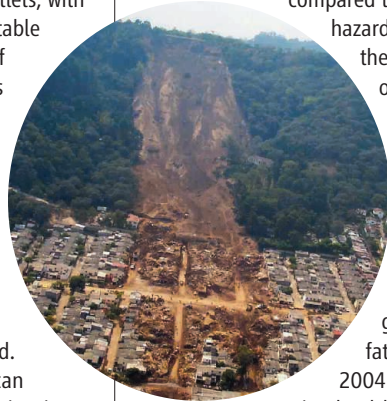
Agriculture is a thirsty business. Despite being grown in the humid tropics, bananas (genus *Musa*) are susceptible to even mild drought and



can require irrigation. A few strains dominate commercial banana production, but much greater banana biodiversity is represented in the Musa International Germplasm collection. Analyzing the genetics driving drought resistance in bananas is challenging, however, because of their growth requirements. To overcome this, Vanhove *et al.* analyzed in vitro banana plantlet growth rates in response to mild osmotic stress. The results

pointed toward variants known to be more tolerant of irregular water availability in field settings. Analysis of leaf proteomes showed differences between stressed and nonstressed plantlets, with most of the proteome variation attributable to a handful of proteins. Annotations of these proteins suggested that pathways involving photodynamic damage and oxidative stress were activated in the osmotic stress response. Placing their work in context, the authors distinguish between drought survival mechanisms and water use efficiency. Plants that use survival mechanisms—such as closing stomata—to withstand drought are likely to show reduced yield. The variants identified here, however, can tolerate temporary and mild water deficiencies without sacrificing plant growth and yield. — PJH

Front. Plant. Sci. **3**,176 (2012).



statistics are useful in calculating risks associated with a particular hazard. Fatalities from landslides, however, have been poorly quantified as compared to those from other hazards, in part because of their concurrence with other events such as earthquakes and tropical cyclones. To reassess the loss of life from landslides, Petley compiled an exhaustive global data set of fatal landslides from 2004 to 2010, excluding landslides triggered by

earthquakes. These 2620 landslides resulted in 32,322 deaths—most occurring in the Himalaya mountains and China—an estimate an order of magnitude larger than those previously drawn from other databases. Because landslides are triggered by increased rainfall and human activities such as environmental degradation, fatalities from landslides may increase with climate change and increased urbanization. — NW

Geology **40**, 10.1130/G33217.1 (2012).

APPLIED PHYSICS

Single Entry

Optical fibers provide the backbone of modern communication networks, with information encoded in the wavelength and polarization states of light pulses that each contain billions of photons. Higher data transmission rates, as well as more secure communication afforded by fundamental laws of quantum mechanics, will require the use of single photons as the information carriers. However, because the emission of single photons from quantum emitters such as quantum dots is generally directionally random, getting the single photons into the fiber remains an engineering challenge. Yalla *et al.* present a relatively simple solution in the form of a tapered optic fiber—a standard optic fiber that has been heated locally and stretched so that it is thinner along one part. They place several quantum dots along the tapered section of the fiber, excite them with an external laser source to emit single photons, and then show that the coupling of the single photons into the fiber can be as high as 20%. Configuring the structure of the tapered fiber provides a flexible route to optimizing the efficient channeling of single photons for communication applications. — ISO

Phys. Rev. Lett. **109**, 63602 (2012).

GEOPHYSICS

Slide Hazards

Quantifying the number of fatalities after natural disasters is a challenging yet critically important task. In the wake of an individual event, response teams use this information to focus immediate rescue efforts. In the longer term, human loss

CELL BIOLOGY

A Close-Up View of Endocytosis

Clathrin-coated pits mediate the uptake of extracellular ligands into cells. Although this process is relatively well understood, questions still remain about how individual clathrin coated pits are initiated and precisely how the budding and scission process to form coated vesicles in the cytosol proceeds. Now, using mammalian live-cell and single-molecule imaging, Cocucci *et al.* provide a close-up view of the initiation process—the first 5 s in the life of a coated pit. Coated pits appeared to be initiated by the coordinated assembly of individual clathrin triskelions together with their AP2 adaptor proteins. The Fcho proteins, known to play important roles in clathrin-coated vesicle assembly, were required for the sustained growth of the incipient coated pits. Working in yeast cells, Kikuluski *et al.* used correlated fluorescence microscopy and electron tomography to look at individual endocytic events to reconstruct a virtual ultrastructural movie of membrane invagination. In this system, the coating of the membrane with clathrin was not sufficient to initiate budding—the actin network was required to promote the formation of invaginated tubules, which were severed from the surface once they had penetrated about 100 nm, which took about 9 s. — SMH

Cell **150**, 495; 508 (2012).

Call for Papers

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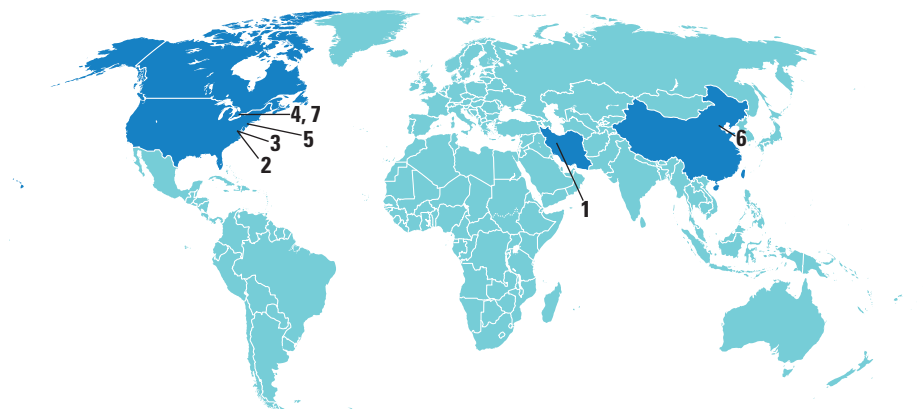
Garret A. FitzGerald, M.D.

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AROUND THE WORLD



Tehran 1

Options Narrow for Iranian Women

Iran's science ministry has forbidden women to study dozens of subjects at 36 universities, according to reports by state-run media. The decree marks a significant erosion of gender-equality measures introduced by former President Mohammad Khatami a decade

ago. "Gender discrimination has been reintroduced," says a scholar in Tehran who requested anonymity.

The move is part of a months-long effort to segregate students by gender in the wake of anti-government protests in 2009. Subjects now off limits to female students

include nuclear physics, petroleum engineering, and English literature. The restrictions came to light earlier this month. Last week, state media noted the latest salvo in the Iran government's campaign: the establishment of 12 women-only hospitals at medical universities.

"There is a lot of fight-back," including picketing on campuses, says the Iranian scholar. But the female students have not yet recaptured any lost ground.

Washington, D.C. 2

Legal Win for Stem Cell Research

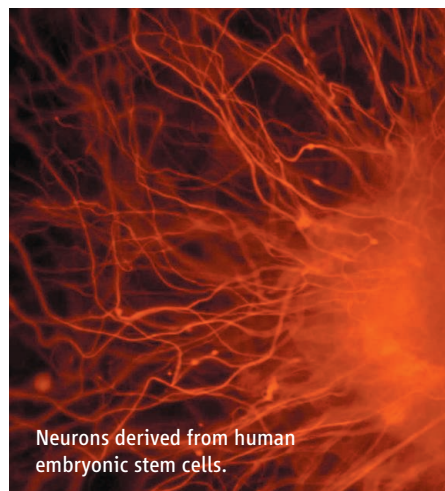
A three-judge panel of a U.S. appeals court ruled last week that federally funded research on human embryonic stem cells

(hESCs) is legal, bringing closer to an end a court battle that has threatened to block hESC research funded by the National Institutes of Health (NIH).

The suit, *Sherley v. Sebelius*, was filed 3 years ago by two scientists who study adult stem cells. They argued that new NIH guidelines easing limits on hESC research violated a 16-year-old law banning federal funds for research in which human embryos are destroyed. On 24 August, Chief Judge David Sentelle of the U.S. Court of Appeals for the D.C. Circuit upheld a lower court's ruling, stating that NIH "had reasonably interpreted" the law to allow for federal funding of hESC studies because research on hESCs and their derivation are separate.

While the other two judges concurred, they did so for different reasons, increasing the chance that the plaintiffs will successfully petition for a new review by the full court, legal experts say. An attorney for the plaintiffs said they are more likely to appeal to the U.S. Supreme Court.

http://scim.ag/stem_cell_law



Neurons derived from human embryonic stem cells.



National Institutes of Health.

Bethesda, Maryland 3

NIH's Millionaires To Get Extra Scrutiny

Researchers with more than \$1 million a year in grants will get extra scrutiny from the U.S. National Institutes of Health (NIH) under a new policy.

The plan is one of several ideas NIH has floated to squeeze more research grants from its flat budget. Applications from principal investigators (PIs) with at least \$1 million a year in direct research funding will get an extra review from the funding institute's scientific council to make sure the research is "both highly promising and distinct from" the PI's other projects, NIH announced on 20 August.

About 89 grants will meet the cutoff for review at September's council meetings. That's less than 1% of all proposals going to the councils, NIH estimates. And because the policy is not a cap, it's unclear how much money it will ultimately free up.

Howard Garrison of the Federation of American Societies for Experimental Biology says the policy is important anyway. "It's not necessarily going to solve all our problems. But people felt it was an appropriate step," Garrison says.

http://scim.ag/NIH_grants

Toronto, Canada 4

Canada's Cash Controversy

On 20 August, the Bank of Canada apologized for expunging an Asian-looking sci-

NOTED

>After landing on Mars, taking its first steps, and testing its rock-zapping laser, on 28 August **the intrepid Curiosity rover also burst into song**. Rapper will.i.am wrote the song, titled "Reach for the Stars." NASA arranged the interplanetary broadcast to correspond with an event about Mars research aimed at grades K-12.

entist from a new \$100 banknote after some Canadians objected to the figure.

The kerfuffle over the image began several years ago when focus groups reviewed the proposed design for a bill highlighting Canada's contributions to biomedical science. Some group members complained that "Asian should not be the only ethnicity represented" and that the image "stereotype[d] ... Asians [as] excelling in technology and/or the sciences," *The Vancouver Sun* reported. The bank redrew the image to appear more Caucasian, a move that has ruffled feathers.

The new plasticized banknotes, which went into circulation this year, are more secure, cheaper, and greener than existing bills. The bank's governor said the bank will not reinstate the original image on the bill, but will review the design process for new currency in light of the ensuing public outcry. <http://scim.ag/CACash>

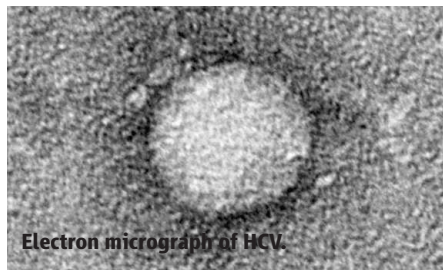
Princeton, New Jersey 5

Promising Hepatitis C Drug Scuttled

Bristol-Myers Squibb announced on 23 August that it had pulled the plug on development of a promising drug that directly attacks the hepatitis C virus (HCV). HCV infects an estimated 160 million people worldwide and causes liver damage. Known as BMS-986094, the drug showed

serious toxic effects when one patient in a clinical trial of the compound died from heart failure and eight others were hospitalized for heart and kidney toxicity. The drug, which was tested on about 250 people, inhibits a nucleotide polymerase that HCV needs to copy itself.

Meanwhile, the U.S. Food and Drug Administration put another nucleotide polymerase inhibitor made by Idenix of Cambridge, Massachusetts, on "partial



Electron micrograph of HCV.

clinical hold" to review safety data. A third drug in this class, GS-7977, made by Gilead Sciences of Foster City, California, has moved furthest along in the development pipeline, and has not raised significant safety concerns. "Developing a drug is like Internet dating," says Tracy Swan, who directs the hepatitis project at the Treatment Action Group, an advocacy group in New York City. "The less you know the better it looks."

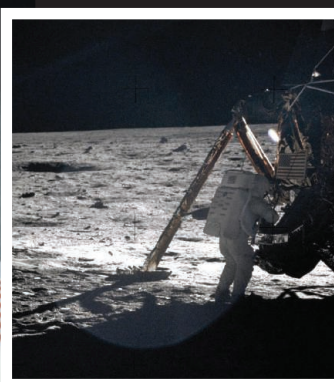
Beijing 6

Cheetah Fossil a Fake

An embarrassing saga for paleontologists and a top science journal has ended. The *Proceedings of the National Academy of Sciences* (PNAS) posted a retraction online on 20 August acknowledging that a cheetah skull—described in a January 2009 report as "the most primitive cheetah known to date"—was a false composite of much older bones.

In the original paper, Ji H. Mazák of the Shanghai Science and Technology Museum and Per Christiansen, then of the Zoological Museum in Copenhagen, described the skull's "unique combination of primitive and derived traits." Only days later, Deng Tao, an early mammals expert at the Institute of Vertebrate Paleontology and Paleoanthropology in Beijing, wrote a letter to PNAS calling the cheetah skull a "fossil forgery." PNAS declined to publish Deng's letter, and Mazák refused to give Deng access to the skull (*Science*, 24 December 2010, p. 1740). Mazák relented last May, allowing Deng to examine the specimen. "I saw that the fossil was very seriously forged," Deng says. Mazák, he says, concurred and signed the retraction. Christiansen, now chief zoologist at Aalborg Zoo in Denmark, told

>>



Winking at the Moon

Astronaut Neil Armstrong, the first person to walk on the moon, died last Saturday at the age of 82. On 20 July 1969, Armstrong and *Apollo 11* spacecraft co-pilot Edwin "Buzz" Aldrin took mankind's first steps on another heavenly object. The mission was Armstrong's third, and last, spaceflight. He left NASA in 1971 and became a professor of aeronautical engineering at the University of Cincinnati in Ohio, where he taught for almost a decade. He spent the rest of his career serving on the board of several large high-tech companies before retiring to a farm in his home state of Ohio. "The next time you walk outside on a clear night and see the moon smiling down at you," his family said in a statement Saturday, "think of Neil Armstrong and give him a wink."

>>AROUND THE WORLD

Retraction Watch that he “had no idea about any of this” and that *PNAS* did not contact him about the retraction.

Canada 7

Arctic Research Station Gets Funded

Last week, Canada announced that it has earmarked more than CAD \$140 million (about US \$141 million) for a new research station in the High Arctic, with an additional CAD \$46 million set aside for the station’s “science and technology program.” The announcement comes only months after another Canadian research station, the Polar Environment Atmospheric Research Laboratory (PEARL), responsible for monitoring polar atmospheric

conditions, was forced to cease year-round operations because of funding cuts. The new facility is 1300 kilometers south of PEARL and cannot adopt its role monitoring changes in air quality, climate, or the ozone. “No one in the [scientific] community has a clue what this [new facility] is going to be used for,” says climate scientist Andrew Weaver of University of Victoria in Canada. The announcement by Canada’s Prime Minister Stephen Harper “is all about sovereignty, gas, and oil exploration, and has absolutely nothing to do with science,” he adds. Construction on the research station located in Cambridge Bay in northwest Canada will start next year. It is scheduled for completion in 2017.

FINDINGS**Silver Lining in Alzheimer’s Trial?**

Alzheimer’s researchers are used to getting bad news from clinical trials, as one promising drug after another has failed to slow

cognitive declines. In that context, Eli Lilly and Company’s announcement on 24 August regarding solanezumab—an antibody that targets β amyloid, the protein fragment that forms pathological clumps in the brains of patients—actually looks somewhat encouraging.

Solanezumab failed to slow cognitive decline in two trials with more than 2000 people with mild to moderate Alzheimer’s disease. However, the company said in a statement, a secondary analysis that combined data from both trials indicated that the drug did slow cognitive decline in people with mild Alzheimer’s disease. Lilly says its plans for solanezumab are still undecided, but it plans to continue an open-label extension study, in which patients from the two recently completed trials can continue to take the drug.

Pharmaceutical companies have invested heavily in anti-amyloid therapies, with largely disappointing results in clinical trials. But because amyloid begins accumulating in the brain decades before memory loss and other symptoms appear, many researchers believe trials have failed because the drugs were given to patients whose disease was already too advanced. <http://scim.ag/alztrial>

Hungry Monkeys Don’t Live Longer

Eating between 10% and 40% less than normal—what’s called calorie restriction—extends life for a range of animals, including mice and nematodes. But testing whether slashing food intake stretches human life

THEY SAID IT

“The sea-ice death spiral, coming during one of the warmest summers in American history, is just one more clear sign of the deepening climate crisis that we ignore at our own peril.”

—Shaye Wolf, climate science director at the Center for Biological Diversity’s Climate Law Institute in San Francisco, California, referring to record low levels of sea ice in the Arctic measured on 26 August.



Brain scans of people with a genetic mutation that causes early onset Alzheimer’s disease.



Oldest Arthropods in Amber

Roughly 230 million years ago, two mites and a midge got stuck in oozing resin from a now-extinct species of conifer tree in the mountains of northeastern Italy. The insects have now earned the distinction of being the oldest arthropods—vertebrates that include insects, arachnids, and crustaceans—ever found preserved in amber. Arthropods have scuttled over Earth’s surface for more than 400 million years, but prior to this discovery the oldest specimens in amber were 130 million years old.

The three amber-bound arthropods were among roughly 70,000 bits of amber (pictured) excavated from an outcrop in the Italian Dolomite Alps. Unlike the midge, the mites are intact, which allowed the team to identify two new species of mites: *Triasacarus fedelei* and *Ampezzoa triassica*. In their study, published on 27 August in the *Proceedings of the National Academy of Sciences*, the researchers report that the mites are the oldest known ancestors of the Eriophyoidea group of mites, which today includes at least 3500 species.



Two 27-year-old male rhesus monkeys. Left: calorie-restricted. Right: control.

span is impractical—so for more than 20 years, rhesus monkeys at two U.S. facilities have been living on lean rations in the name of science. This week in *Nature*, researchers report that the monkeys at the National Institutes of Health Animal Center in Dicker-son, Maryland, did reap some benefits, such as reduced triglycerides in the blood and a lower cancer rate. But dieting animals aren't living longer than monkeys that ate more.

However, 3 years ago, researchers revealed in *Science* (10 July 2009, p. 201) that the other group of hungry monkeys—at the Wisconsin National Primate Research

Center in Madison—did live longer on their reduced diets. The exact reason for the discrepancy isn't clear, as the two groups of monkeys differ in several ways, including the composition of their diet.

http://scim.ag/monkey_cal

Science LIVE

Join us on Thursday, 6 September, at 3 p.m. EDT for a live chat on **head injury and trauma in soldiers and athletes**.
<http://scim.ag/science-live>

BY THE NUMBERS

\$78.6 million Cost of a new trial by the National Heart, Lung and Blood Institute, part of the National Institutes of Health, to test whether an anti-inflammatory medication prevents cardiovascular disease.

964 Number of measles cases in England and Wales in the first 6 months of 2012, up from 497 in the same period last year.

58% Percentage of likely American voters who think that Indian and Chinese schools are catching up to, or have surpassed, K–12 schools in America, according to a survey of 1227 adults released on 21 August by nonprofit organization The Center for the Next Generation.

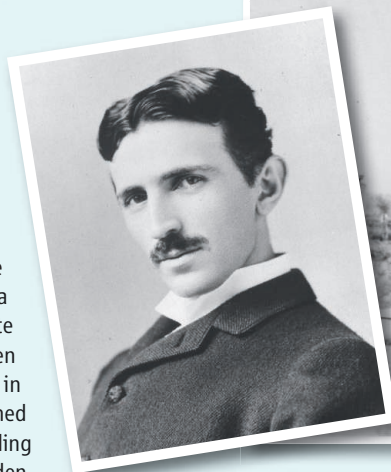
Random Sample

Sparks Fly Over Tesla Museum

Nikola Tesla is credited with inventing wireless transmission, AC power, fluorescent lighting, and other essentials of modern life—yet in the United States, he is still largely unsung. Now, an Internet fundraiser has raised more than \$1 million to build a museum to honor the eccentric Serbian-American scientist.

For nearly 2 decades, organizers of the Tesla Science Center at Wardenclyffe—a nonprofit organization seeking to promote Tesla's contributions to science—have been eyeing the approximately 6-hectare site in Shoreham, New York, where Tesla (left) owned a lab from 1901 to 1915. A futuristic building with a transmission tower (right), the Wardenclyffe lab was supposed to transmit radio and power wirelessly to the world, but it was never completed. Ownership of the site went to the Agfa Corp., which recently decided to sell. The Tesla Science Center pounced on the purchase, says the center's president, Jane Alcorn. "We realized the opportunity was now."

Fundraising for the museum took off when Alcorn got an e-mail from Matthew Inman, creator of the Internet cartoon *The Oatmeal* (<http://theoatmeal.com>). Inman's popular cartoon "Operation Let's Build a



Goddamn Tesla Museum" raised the required \$850,000—which will be matched by New York state—to secure the Wardenclyffe site in less than a week. The 22,000 donors included founders of the electric car company Tesla Motors.

Now that it has purchased the land, the Tesla Science Center is raising money to build the museum itself (<http://igg.me/p/204900>). "Tesla had extravagant dreams, and a wonderfully out-of-the-box kind of imagination," Alcorn says. "We want it to be dramatic and inspiring."



ANCIENT DNA

A Crystal-Clear View Of an Extinct Girl's Genome

Three years ago, German postdoc Matthias Meyer set out to develop a new method for preparing DNA from fossils. Most techniques adapt tools used for sequencing DNA from living humans, but Meyer started from scratch, tailoring his approach to the maddening peculiarities of degraded DNA tens of thousands of years old. While he worked long hours in the lab, other researchers used standard sequencing methods to produce the first genomes of two archaic humans, albeit of low quality. Meyer almost gave up. But now, in a stunning technical feat, he and colleagues at the Max Planck Institute for Evolutionary Anthropology in Leipzig, Germany, have sequenced the genome of an archaic Siberian girl 31 times over, using a new method that amplifies single strands of DNA.

As the international team reports in a paper published online in *Science* this week, more than 99% of the nucleotides are sequenced at least 10 times, so researchers have as sharp a picture of this ancient genome as of a living person's. "No one thought we would have an archaic human genome of such quality," Meyer says. "Everyone was shocked by the counts. That includes me."

That precision allows the team to compare the nuclear genome of this girl, who lived in Siberia's Denisova Cave more than 50,000 years ago, directly to the genomes of living people, producing a "near-complete" catalog of the small number of genetic changes that

make us different from the Denisovans, who were close relatives of Neandertals. "This is the genetic recipe for being a modern human," says team leader Svante Pääbo, a paleogeneticist at the institute.

Ironically, this high-resolution genome means that the Denisovans, who are represented in the fossil record by only one tiny finger bone and two teeth, are much better known genetically than any other ancient human—including Neandertals, of which there are hundreds of specimens. The genome offers a glimpse of what the Denisovan girl looked like—her eyes, hair, and skin were brown—and new details about how her lineage evolved. The team confirms that the Denisovans interbred with the ancestors of some living humans and found that Denisovans had little genetic diversity, suggesting that their small population waned further as populations of modern humans expanded. "Meyer and the consortium have set up the field of ancient DNA to be revolutionized—again," says Beth Shapiro, an evolutionary biologist at the University of California, Santa Cruz, who was not part of the team. Evolutionary geneticist Sarah Tishkoff of the University of Pennsylvania agrees: "It's really going to move the field forward."

Pääbo's group first gave the field a jolt in May 2010 by reporting a low-coverage

Gene jockey. Matthias Meyer developed a new method to prepare single strands of ancient DNA.

sequence (1.3 copies on average) of the composite nuclear genome from three Neandertals. They found that 1% to 4% of the DNA of Europeans and Asians, but not of Africans, was shared with Neandertals and concluded that modern humans interbred with Neandertals at low levels (*Science*, 7 May 2010, pp. 680, 710).

Just 7 months later, the same group published 1.9 copies on average of a nuclear genome from a girl's pinky finger bone from Denisova Cave. They found she was neither a Neandertal nor a modern human—although bones of both species had been found in the cave—but a new lineage that they called Denisovan (*Science*, 28 January 2011, p. 392). The team found "Denisovan DNA" in some island Southeast Asians and concluded that their ancestors also interbred with the ancestors of Denisovans, probably in Asia.

But these genomes were too low quality to produce a reliable catalog of differences. Part of the problem was that ancient DNA is fragmentary, and most of it breaks down into single strands after it is extracted from bone.

Meyer's breakthrough came in developing a method to start the sequencing process with single strands of DNA instead of double strands, as is usually done. By binding special molecules to the ends of a single strand, the ancient DNA was held in place while enzymes copied its sequence. The result was a sixfold to 22-fold increase in the amount of Denisovan DNA sequenced from a meager 10-milligram sample from the girl's finger. The team was able to cover 99.9% of the mappable nucleotide positions in the genome at least once, and more than 92% of the sites at least 20 times, which is considered a benchmark for identifying sites reliably.

About half of the 31 copies came from the girl's mother and half from her father, producing a genome "of equivalent quality to a recent human genome," says paleoanthropologist John Hawks of the University of Wisconsin, Madison, who was not part of the

team. Shapiro calls the new method "spectacular. ... It's exactly that very simple, incredibly good idea that makes you kick yourself for not coming up with it first."

In fact, others had thought of the same approach. But "it ain't easy," demanding lots of time and money, says geneticist Hendrik Poinar of McMaster University in Hamil-

Online

sciencemag.org

Podcast interview with author Ann Gibbons (http://scim.ag/pod_6098).

ton, Canada. His lab has tried to copy single strands, with “modest” results.

Now, the view of the ancient genome is so clear that Meyer and his colleagues were able to detect for the first time that Denisovans, like modern humans, had 23 pairs of chromosomes, rather than 24 pairs, as in chimpanzees. By aligning the Denisovan genome with that of the reference human genome and counting mutations, the team calculated that the Denisovan and modern human populations finally split between 170,000 and 700,000 years ago. (The range of error is large because of uncertainty in the average human mutation rate, lately the subject of intense debate and a flurry of papers.)

The researchers also estimated ancient Denisovan population sizes by using methods to estimate the age of various gene lineages and the amount of difference between the chromosomes the girl inherited from her mother and father. They found that Denisovan genetic diversity, already low, shrank even more 400,000 years ago, reflecting small populations at that time. By contrast, our ancestors’ population apparently doubled before their exodus from Africa.

The team also counted the differences between Denisovans and chimps, and found that they have fewer differences than do modern people and chimps. The girl’s lineage had less time to accumulate mutations, and the “missing evolution” suggests she died about 80,000 years ago, although the date is a “best estimate” and, therefore, tentative, says co-author David Reich, a population geneticist at Harvard University. If this date—the first proof that a fossil can be directly dated from its genome—holds up, it is considerably older than the very rough dates of 30,000 to more than 50,000 years for the layer of sediment where the fossils of Denisovans, Neandertals, and modern humans all were found. “It’s great that you can start to put a genetic date on the fossil, because we don’t have any decent absolute dates for these fossils,” says paleoanthropologist Chris Stringer of the Natural History Museum in London.

Did the ancestors of Europeans and Asians really mix it up with Denisovans and Neandertals? Some researchers have questioned that conclusion of the first two archaic genome papers. “Introgression makes no sense to me,” says paleoanthropologist Richard Klein of Stanford University in Palo Alto, California. The population models used in the 2010 analyses of the Neandertal genome, for example, could not rule out the possibility that the archaic DNA in modern

genomes comes from a different source in Africa rather than introgression with Neandertals and Denisovans. The scenario, originally proposed by co-author Montgomery Slatkin of the University of California, Berkeley, is that there were two distinct populations in east and west Africa. One gave rise to Neandertals and modern humans, who left east Africa carrying very ancient pieces of DNA from these ancestors. The other group gave rise to modern sub-Saharan Africans, who lack those particular ancient motifs. In a paper earlier this month in the *Proceedings of the National Academy of Sciences*, evolutionary biologist Andrea Manica of the University of Cambridge in the United Kingdom wrote that this alternative scenario cannot be ruled out



Slice of life. This replica of a tiny finger bone from Denisova Cave (right) yielded an entire genome.

without genomes from many different Africans and more detailed population models.

But new analyses make this alternative idea almost impossible and suggest that our ancestors did indeed interbreed at least twice with archaic peoples, Slatkin says. Given the Denisovan DNA in Southeast Asia, it’s almost impossible to model a scenario where both the Neandertal and Denisovan DNA are inherited from ancient Africans, because the DNA would have to persist unchanged over hundreds of thousands of years, he says. And in a paper in press in *PLoS Genetics*, Reich’s group calculates that Europeans inherited this “archaic” DNA 37,000 to 86,000 years ago—too recent to be from ancient Africans.

The team says the new genome confirms their previous findings, showing that about 3% of the genomes of living people in Papua New Guinea come from Denisovans, while the Han and Dai on mainland China have only a trace of Denisovan DNA. Furthermore, the team determined that Papuans have more Denisovan DNA on their autosomes, inherited equally often from both parents, than on their X chromosomes, inherited twice as often from the mother. This curious pattern suggests several possible scenarios, including that male

Denisovans interbred with female modern humans, or that these unions were genetically incompatible, with natural selection weeding out some of the X chromosomes, Reich says.

The new genome also suggests one odd result. By using the detailed Denisovan genome to sharpen the view of their close cousins the Neandertals, the team concludes that living East Asians have more Neandertal DNA than Europeans have. But most Neandertal fossils are from Europe; Klein calls the result “peculiar.”

Most exciting to Pääbo is the “nearly complete catalog” of differences in genes between the groups. This includes 111,812 single nucleotides that changed in modern humans in the past 100,000 years or so. Of those, eight



were in genes associated with the wiring of the nervous system, including those involved in the growth of axons and dendrites and a gene implicated in autism. Pääbo is intrigued in particular by a change in a gene that is regulated by the so-called *FOXP2* gene, implicated in speech disorders. It is “tempting to speculate that crucial aspects of synaptic transmission may have changed in modern humans,” the team wrote. Thirty-four genes are associated with disease in humans. The list suggests some obvious candidates for gene-expression studies. “The cool thing is that it isn’t an astronomically large list,” Pääbo says. “Our group and others will probably be able to analyze most of them in the next decade or two.”

Back in Leipzig, the mood is upbeat, as researchers pull fossil samples off the shelf to test anew with “Matthias’s method.” First on Pääbo’s list: Neandertal bone samples, to try to produce a Neandertal genome to rival that of the little Denisovan girl.

—ANN GIBBONS

PUBLIC HEALTH

Outbreak Pattern Stymies Vaccine Work

West Nile virus has been a worry for U.S. public health departments for more than a decade, but the outbreak this year could be on track to be the country's largest ever. It has already prompted a blitz of warnings to avoid virus-infected mosquitoes, underlined with a caveat: There is no vaccine or treatment for West Nile infection. Researchers say the failure is not for lack of trying.

After the first cases were detected 13 years ago in New York City, a burst of money went into thwarting the then-mysterious disease. The basic science has come a long way since then, but measures to prevent or treat West Nile infection have stalled. One big reason is that the sporadic nature of outbreaks makes it hard to get enough volunteers for trials.

The peak so far for West Nile was 2003: 9862 cases and 264 deaths were reported to the Centers for Disease Control and Prevention (CDC). This year, as of 21 August, CDC had received reports of 1118 cases and 41 deaths, the highest count ever by the third week in August. About half the cases have been in Texas, where Dallas declared a state of emergency and began aerial spray-

Although the causes of the outbreak aren't clear, many experts suspect that hot weather and drought combined to spur an explosion in *Culex* mosquitoes, the species that spreads West Nile, and a high rate of virus-infected mosquitoes. In addition, a mild winter helps overwintering adult mosquitoes survive. Hot temperatures spur mosquito development and allow the virus to replicate faster inside mosquitoes, notes arbovirologist Laura Kramer of the New York State Department of Health in Albany. And drought favors *Culex* mosquitoes' preferred breeding habitat: pools of water rich in organic material, such as in underground drains. Normally, rains flush out such pools. In addition, drought may have forced birds that carry and amplify the virus to move from rural areas to cities to find water, says entomologist William Reisen of the University of California, Davis.

Much of the virus's transmission cycle is now well-known: "I think we've dissected it more than any other mosquito-borne virus that's been studied," Kramer says. Researchers also developed fast diagnostic tests for West Nile, including one that has protected the blood supply. However, efforts to develop a licensed vaccine stalled. The National Institute of Allergy and Infectious Diseases (NIAID) poured money into a vaccine made from a weakened backbone of yellow fever virus with two genes for coat proteins swapped for the West Nile version of those genes. The vaccine, developed by Acambis in Cambridge, Massachusetts, made it through a phase II trial that found a single dose was safe and could generate high levels of protective antibodies.

"This is a really good vaccine," says Thomas Monath, former Acambis vice president and now a consultant.

But in 2008, Sanofi Pasteur bought Acambis and suspended the West Nile program. West Nile cases had dropped from 2006 to 2008, and the company decided to focus on other priorities, including a dengue

vaccine, says Sanofi spokesperson Susan Watkins. "Unfortunately we have to pick and choose, and it didn't make the cut."

Some researchers say market uncertainty may have been a problem, too. "Who's going to pay for a vaccine? It won't be cheap," says virologist Robert Tesh of the University of Texas Medical Branch in Galveston. It might be given only to the elderly, who are most vulnerable to severe disease, and perhaps highly exposed groups such as outdoor workers, says CDC's Erin Staples. Monath, however, points to new evidence suggesting that West Nile virus can cause kidney damage in younger people. A team at Baylor College of Medicine in Houston, Texas, reported last month in *PLoS ONE* that in a cohort of 139 people with a mean age of 57 who tested positive for West Nile virus, years later 40% had evidence of kidney disease. "If proven, this creates a completely different set of priorities for a vaccine," Monath says.

Work on treatments has been delayed for similar reasons. Trials of three drugs—a monoclonal antibody, interferon, and immunoglobulin—were halted in part because researchers couldn't enroll enough trial volunteers, Staples notes. "It's going to be difficult to do any kind of study with patients in outbreak sites when you don't know when and where it's going to occur," says Patricia Repik of NIAID.

Yet even if treatments were available, they might not be of much help. The virus usually causes only flulike symptoms at first; less than 1% of those infected develop serious neuroinvasive disease. By the time patients reach a hospital, often with meningitis or encephalitis, "the nerve damage is already done," Tesh says. What's needed, Repik says, is "almost a home test" so people who get sick could know early on whether they have a West Nile infection. The National Institutes of Health is funding such research as part of \$65 million it will spend this year on West Nile and related diseases.

One frustration for West Nile experts is that funding for state labs and CDC's vector-borne diseases division has dropped off in recent years. Kramer predicts, "This year may change that." At the same time, West Nile infections will likely subside next year in the current epidemic areas because so many birds will have become infected and will be immune to the virus, Reisen says.

—JOCELYN KAISER



Last resort. Dallas, Texas, undertook nighttime chemical spraying to knock down the population of virus-carrying *Culex* mosquitoes.

ing. Other hot spots are in Mississippi, Louisiana, South Dakota, and Oklahoma. Given the up to 2-week lag between when people become infected and when they become ill, cases will likely keep rising through September, says Lyle Petersen, director of CDC's division of vector-borne diseases in Colorado.

RESEARCH QUALITY

Service Offers to Reproduce Results for a Fee

Spotting a business opportunity and a chance to change the culture of science, a breast cancer biologist is hoping to persuade researchers to have their work replicated for a fee. They would accept the risk of failure but also have a shot at quick validation.

The Reproducibility Initiative, launched earlier this month by Elizabeth Iorns in Palo Alto, California, invites biomedical scientists to submit critical experiments to an advisory board, which matches those experiments with a research facility equipped to repeat them. The original author—and hopefully everyone else—can learn in a short time whether new research holds up. The journal *PLoS ONE* has pledged to publish any work that comes out of the Reproducibility Initiative.

As with any novel venture, the initiative comes with looming questions. One is how many will participate. The ability to entice scientists will depend partly on cost. For the moment, applicants must cough up the cost of replication, estimated at about 10% of their original study, which could easily run into the thousands of dollars. (The initiative takes a 5% cut of that payment.) Iorns's group has received three applications so far.

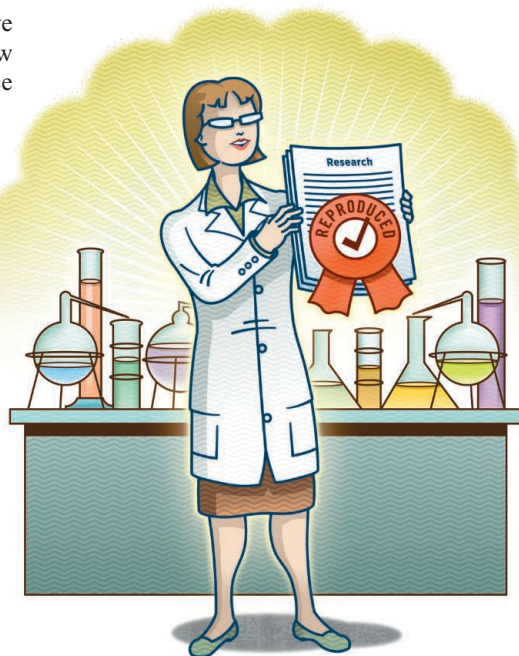
Cost and the difficulty of getting a publishable paper from repeating experiments often make replication impractical for scientists. "People are really very poorly incentivized to check out whether other people's work is right," says Hal Pashler, a cognitive psychologist at the University of California, San Diego. In January, Pashler set up PsychFileDrawer, a mechanism whereby psychologists can post replications. Despite plenty of visitors curious to see whether a colleague's work has been thrown into question, the site lists only about 13 experiments.

Because the Reproducibility Initiative is asking scientists to put their own and not others' work to the test, it's taking pains not to alienate potential applicants. Its focus, Iorns says, is on celebrating research that is successfully reproduced. Work that's not replicated the first time can be sent to another lab for a second round. Iorns chooses her words carefully when describing what a double failure means. "It doesn't necessarily invalidate the experiment," she says. "It indicates you have a robustness problem."

The results go back to the scientist, who

will be a co-author of a paper reporting the replication, even though he or she did not conduct those experiments. The original author also decides whether to publish what the replicators produced. *PLoS ONE* will peer-review the studies, but because the journal accepts all papers that are technically sound, Iorns can't imagine it would turn down one from the initiative.

Iorns considers the 10% estimate realistic because only a subset of the original study will be repeated. Submitters provide their methods and results; those overseeing the Reproducibility Initiative home in on the experiments that seem most germane to the paper's conclusion, minimizing the number of animals needed, for example.



Cost is unquestionably a hurdle. "Many people would not be able to afford" what's charged, or "would choose not to spend their money that way," Pashler says. Iorns says some funders "have committed to funding the replications of their researchers," but she can't share yet who they are. Industry money is also an option. "We'll need capital to scale, and that capital has to probably come from the industry side of the equation," says Bruce Booth, a member of the initiative's advisory board. Booth is a partner at Atlas Venture, a venture capital firm in Cambridge, Massachusetts.

Another question is whether most biomedical research can or even should be replicated

by Iorns's system. She plans to have the labor done through Science Exchange, a match-making service she founded last year as a way to outsource certain types of research, such as gene sequencing. Industry or academic researchers pay a fee to one of more than 1000 "Core Facilities," mainly in universities, who do the work for them. Replicators will not be told the outcome of the original experiment to guard against bias, although in theory they could look up the publication they're tasked with replicating.

"Not any laboratory could turn around and do something that you've been spending most of your career learning," says Ferric Fang, a microbiologist at the University of Washington, Seattle, and editor-in-chief of the journal *Infection and Immunity*. Fang is finishing a paper now that he's been working on for 12 years and says it's hard for him to imagine many scientists with the expertise to repeat those experiments.

At the same time, Fang acknowledges that Iorns is putting her finger on a problem: The scientific literature is replete with studies that don't hold up. In March, a paper in *Nature* reported that Amgen scientists had attempted to replicate 53 important cancer experiments and only six had panned out.

Iorns's effort comes as the reproducibility problem is gaining traction at various levels. In June, the Association for Psychological Science and the U.S. National Institutes of Health held a meeting to discuss replication of behavioral research; Pashler attended, as did social psychologist Brian Nosek of the University of Virginia in Charlottesville.

Nosek, who is also an adviser to the Reproducibility Initiative, has recruited 72 of his colleagues so far to try to replicate dozens of studies from papers published in 2008—a random sampling that aims to assess the reproducibility of psychological science. Most of the studies are inexpensive to repeat, and labs are paying for them out of their own pocket. Iorns hopes that her initiative will accomplish something similar despite high hurdles. "If you're an academic researcher ... and you want to stand out, you want to say, 'I'm not part of the problem' " of research that can't be replicated, she says. That, she believes, will bring people her way.

—JENNIFER COUZIN-FRANKEL

In the Hunt for the Red Planet's Dirtiest Secret

More obstacles to Curiosity rover's search for the organic remains of martian life are turning up, complicating an already daunting task

DISCOVERING THE ORGANIC REMAINS OF life can be dirt-simple, at least on Earth. Kneel in any reasonably productive garden and your pants will carry the dark stains—organic matter—of long-dead and decomposed tomatoes, basil, and earthworms.

Finding signs of life that thrived eons ago is an entirely different matter. Just ask geologist John Grotzinger. After 20 years of fieldwork roaming across the Precambrian rock of Namibia, he had found only three new fossil deposits. “It’s actually hard to find fossils,” Grotzinger says, “and that’s on Earth. This is really just hard to do.”

But now as project scientist on the Curiosity rover mission, Grotzinger and his 400-strong science team are taking on a far greater challenge: searching 3-billion-year-old rock at a single spot on an alien planet for the organic remains—molecular fossils, if you will—of life. Finding such organic mat-

ter on Mars “is just going to be very, very hard to do,” Grotzinger says.

And as Curiosity cruised toward Mars and a safe landing on 6 August (EDT), researchers were recognizing yet more ways that Mars could be destroying organic evidence of any past life. Megajoule cosmic rays blast organic molecules to bits, and a chemically reactive brew in martian soil probably chews up organic matter in a few millennia, never mind eons. Given the dearth of studies of organic matter’s fate under martian conditions, Curiosity’s chances of success are anyone’s guess.

0 for 3, so far

Three times, NASA has sent spacecraft that could detect organic matter to the surface of Mars. And three times, martian soil has yielded no organics. All three of those landers—Viking landers 1 and 2 in 1976 and the Phoenix lander in 2008—carried analytical systems with the same basic design as Curiosity’s. A soil sample is heated to hundreds of degrees, driving off volatile organics or breaking down nonvolatile organics into fragments that can also be driven off. In Viking, the volatilized organics were separated from one another as they passed through a gas chromatograph. In all three, a mass spectrometer then identified each organic compound by its mass.

But even though the Viking landers were sensitive enough to detect organics in concentrations of a few parts per billion, they found nothing more than trace amounts of two small chlorine-containing hydrocarbons. Those were identified as contaminants. Phoenix produced some carbon dioxide, but team

members concluded that it probably came from the breakdown of inorganic carbonate, not organics.

The nondetection of martian organics was quite a puzzler. Everyone knew that something like 1000 tons of organic matter gets dumped on Mars every year as organic-rich cosmic dust—debris from comets and asteroids—sifts to the surface. Left to accumulate, that much organic matter would lead to soil concentrations of tens of thousands of parts per million, not sub-part-per-billion. The missing organic matter prompted much talk of a “superoxidizer” in martian soil, a chemical that could destroy organics by adding oxygen to them and eventually converting them entirely to carbon dioxide.

The Viking landers were not designed to measure oxidizers, but plenty of candidates were put forward. They included hydrogen peroxide produced by solar ultraviolet hitting the atmosphere, nanophase iron in minerals, and oxidizers produced when whirling dust devils electrically charge up the atmosphere, among others.

Looking bleak

Thirty years later, Phoenix finally detected a bona fide oxidizer in martian soil: perchlorate salts likely produced in the atmosphere. Perchlorate—a combination of a chlorine atom and four oxygen atoms that can pair with a metallic atom like magnesium—won’t oxidize much of anything if left to itself at martian temperatures. But hit it with enough energy and it can do plenty of damage.

One way to energize perchlorates would be to irradiate them. Perchlorates on Mars are continually bombarded by galactic cosmic rays that can decompose them into potent oxidizers like hypochlorite (the active component of household bleach). Geo-



The complicator. The Phoenix lander discovered martian perchlorate that, once cosmic rays decompose it, destroys organic matter.

On to organics? By far the most promising site for Curiosity rover's search for life's organic matter is the base of 5-kilometer-high Mount Sharp.

chemist Richard Quinn of NASA's Ames Research Center in Mountain View, California, and his colleagues created something like martian irradiation conditions in the lab by bombarding magnesium perchlorate with x-rays that, like cosmic rays, can decompose perchlorates. As they reported at the 2011 Lunar and Planetary Science Conference, Mars-like irradiation produced four or five chlorine-containing breakdown products, including hypochlorite.

By including irradiated perchlorates in lab reruns of two Viking lander experiments, Quinn and his colleagues showed that perchlorates also likely yield strong oxidizers on Mars. They found that irradiated perchlorate released oxygen gas when moistened, just as soil samples did in Viking's Gas Exchange experiment. And when irradiated perchlorate was moistened with a solution containing the organic compound formate, carbon dioxide containing the formate's carbon was generated, just as happened in Viking's Labeled Release experiment.

But degraded perchlorate is not the only emerging threat to martian soil organics. Cosmic rays themselves destroy organic matter. So planetary scientist Alexander Pavlov of NASA's Goddard Space Flight Center in Greenbelt, Maryland, and his colleagues calculated as best they could how fast cosmic rays might be destroying organics on Mars. "The problem is that very little experimental work has been done" on how quickly cosmic rays can degrade organics, Pavlov says. But they took one experimentally determined destruction rate for amino acids reported in the literature and combined it with the flux of cosmic rays expected to pass unimpeded through the vanishingly thin martian atmosphere and penetrate a meter or two into surface rock.

As the group reported in a *Geophysical Research Letters* paper published on 7 July, the prospects are not good for finding big, complex organic molecules—the hallmark of once-living organisms—near the surface. Cosmic-ray irradiation "is a problem if you're going for something billions of years old only to [a depth of] 10 centimeters," Pavlov says—about the depth soil samples can be scooped. Small organics have a longer lifetime, he explains, because they present a smaller target for cosmic rays, "but they're not going to be definitive evidence for life." And that's a conservative prognosis. Destruction rates in martian soil are likely to be faster—possibly 100 times faster—than the laboratory rates determined in the absence of minerals, the group writes. "That will pose a serious challenge for organic detection."

And then there is the ultraviolet radiation. Because the atmosphere of Mars lacks protective ozone, the sun's ultraviolet strikes the surface at full force. Although it penetrates rock less than a millimeter, that is enough to destroy the organic matter of incoming cosmic dust, according to a study published on 17 August in two papers in the *Journal of Geophysical Research—Planets*.

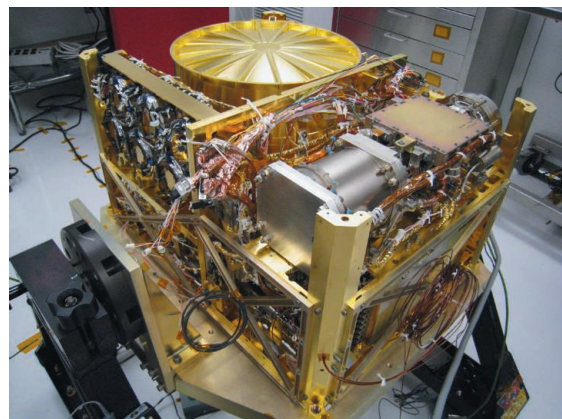
Planetary scientists John Moores of the University of Western Ontario in London, Canada; Andrew Schuerger of the University of Florida, Gainesville (working at the Kennedy Space Center); and colleagues exposed finely ground, organics-rich meteorite to martian surface conditions, including the ultraviolet radiation. They then applied their observed rate of decomposition to the expected properties of the cosmic dust settling onto Mars. They calculate that ultraviolet radiation would destroy half of the cosmic dust's organic matter in about a millennium and in several millennia it would all be gone.

Organic hide and seek

With decomposed perchlorates, cosmic rays, and ultraviolet radiation ganging up on martian organic matter, Curiosity's chances of finding it when it scoops up its first soil samples are looking slim. And "if we find [soil] organics, it almost certainly will have nothing to do with life," says astrobiologist Christopher McKay of NASA Ames. The most likely organics in soils would be those of cosmic dust because they are continually resupplied, so "detecting organics is not detecting life," he says.

To maximize the chance of finding ancient life's molecular remains intact, Curiosity will have to go for solid rock, and not just any rock, Pavlov says. Unlike its predecessors, Curiosity carries a drill, which can retrieve 10-centimeter-long cores from solid rock. But centimeters of protection won't be enough, Pavlov says: "Our paper calls for being very smart about where you sample." Rather than drilling a geologically enticing outcrop that may have been irradiated by cosmic rays for eons, Pavlov argues for sampling rock exposed only in the geologically recent past, say by being recently excavated by a small meteorite impact.

To up the odds even more, Curiosity has been sent to Gale crater. Grotzinger would be surprised if the enclosed bowl of Gale, one of the lowest spots on the planet, did not hold a lake in ancient times. On Earth, lakes are prime spots for both producing and preserving organic matter. And Curiosity's target rocks at the base of Gale's central mound are layered with clays, water-altered minerals



Lab in a box. With three instruments connected by 600 meters of wiring, Curiosity's microwave-oven-size SAM can analyze the chemical and isotopic makeup of martian soil, rock, and atmosphere.

that can protect organic matter from chemical degradation, especially if quickly buried by more sediment.

And then there is SAM. The Sample Analysis at Mars instrument package will analyze soil and rock samples collected by Curiosity. Although it includes the same basic components as Viking landers and Phoenix, SAM "is a powerful suite of instruments," says McKay, who is on the SAM team. For example, SAM can chemically process samples to prevent perchlorates from destroying organics during the analysis, as McKay suspects happened during Viking. "We have a lot more flexibility and adaptability to explore," he says. He and the rest of Curiosity's 400 are going to need it.

—RICHARD A. KERR

IMMUNOLOGY

The New View of Complement

This cascade of immune proteins has more diverse roles, and can cause more problems, in the body than once thought

If you visited immunologist William Robinson's lab at the Stanford University School of Medicine in Palo Alto, California, a couple of years ago, you might have found his postdoc Qian Wang operating on the knees of mice, performing the same surgery that many athletes undergo to repair a torn meniscus. No, the animals hadn't hurt themselves by running too vigorously on their wheels. Instead, the researchers were testing an unorthodox hypothesis about the cause of osteoarthritis (OA), the painful and sometimes crippling joint degeneration that strikes many of us as we age.

The standard explanation for OA attributes it to the gradual erosion of our joints over decades, but there have long been hints that something else is involved. The autoimmune disease rheumatoid arthritis, another condition that impairs the body's joints, stems from inflammation triggered by the immune system, and the joints of OA patients often show milder inflammation. Researchers haven't been sure, however, whether inflammation drives the damage of OA or is a byproduct of it.

To find out, Robinson's team began operating on multiple strains of genetically engineered mice that lacked various

inflammation-promoting genes, carving away some of their knee cartilage because that typically induces OA. (Athletes who have meniscus surgery frequently develop the arthritis.) The researchers got a jolt when they performed the surgery on mice lacking genes for the complement system, a cadre of immune proteins that researchers didn't think was a factor in OA. Rodents lacking either of two complement proteins incurred about 50% less knee damage than did control animals. And as the team reported last December in *Nature Medicine*, mice missing a complement-inhibiting protein showed more severe erosion. A role for complement in OA is an intellectual leap. "Everyone thinks that OA is simple wear and tear in the joint," Robinson says. "Complement may play a crucial role in the breakdown of cartilage and destruction of the joint in OA."

Arthritis researchers aren't the only scientists recently taken aback by new insights into the complement system. Again and again, it has confounded expectations, proving to be more versatile and powerful than anyone thought. Not that long ago, most researchers agreed that, as its name suggests, complement was a mere helper for immune cells. But then further research demonstrated

that the system is one of our most important protections against pathogens, killing invaders before other immune defenses have a chance to mobilize.

Even more unexpected, some researchers say, is the increasing evidence that complement components perform functions outside the immune system. Complement takes part in the body's growth and maintenance, for example. Recent work suggests it guides development of the brain and skeleton and spurs damaged organs to repair themselves. "The term 'complement' is a misnomer," says immunologist John Lambris of the University of Pennsylvania's Perelman School of Medicine.

But if complement does a lot of good in the body, it can also do us harm. "It's an essential component of normal physiology and pathophysiology," says Lambris, who notes that researchers have implicated the system in more than 30 illnesses. The list includes diseases and conditions known to have an immune connection—such as sepsis, rheumatoid arthritis, and organ transplant rejection—and ones that scientists didn't consider immune system diseases, such as OA and age-related macular degeneration (AMD), the leading cause of blindness for older people in

CREDIT: NATIONAL EYE INSTITUTE, NATIONAL INSTITUTES OF HEALTH

Stealing vision. Drusen, the small, milky blotches on the retina of a patient with age-related macular degeneration, carry proteins from the complement system.

the developed world. The recent work “redefines these degenerative diseases as having a significant immune component and opens up new avenues for treatment,” Robinson says.

Already, two drugs have been approved for complement-related diseases, and several other compounds targeting proteins in the cascade are in clinical trials for a variety of conditions. “That’s pretty remarkable, and it’s just the beginning,” says immunologist John Atkinson of Washington University School of Medicine in St. Louis, who has studied complement for more than 40 years.

Standing guard

Complement belongs to the innate arm of the immune system. Unlike the adaptive immune system that includes B and T cells, the innate arm doesn’t for the most part customize its defenses for specific pathogens. Complement was one of the first immune defenses recognized; at the end of the 19th century, researchers discovered that blood serum contained a bacterium-killing component in addition to antibodies. Complement was also one of the earliest defenses to evolve: Only vertebrates can muster B cells and T cells, but even sponges boast complement proteins, Lambris notes.

Complement usually leads the body’s counterattack against bacteria. In contrast to the adaptive immune system, which can take days or even weeks to reach peak performance, complement is always ready for action, and it dispatches invaders swiftly. “It’s an amazing first responder,” Atkinson says. “It can lyse a bug in 30 seconds.”

A sign of complement’s importance to our survival is that it accounts for about 4% of the proteins in our blood. Among the more than 30 types of complement proteins are danger detectors, activators that switch on other proteins, and inhibitors that curb self-directed attacks. They fall into three interconnected pathways (see figure). Those proteins on the lookout for potential threats are constantly checking the blood and scanning the surfaces of our cells. The complement system has several options once it detects a pathogen or other danger. In the most dramatic response, the complement component C5b and other

proteins convene to form a membrane attack complex, which lands on the surface of a microbe and pierces its membrane.

Stimulating complement can also spur defensive cells such as macrophages to eat an intruder and crank up inflammation, another protective measure. Complement is so good at its job, says immunochemist Robert Sim of the University of Oxford in the United Kingdom, that you usually aren’t aware it’s working; it kills off invading bacteria before they have the opportunity to make you sick.

Another of complement’s crucial roles involves searching out dying body cells and molecular junk. Complement proteins tag but don’t remove the refuse—they hail a macrophage or other cell to clean up—and autoimmune diseases such as lupus may result from the failure of complement to help eliminate this debris.

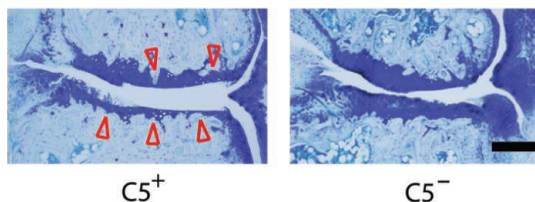
Growth and regrowth

Keeping the body safe is complement’s traditional job, so tidying up potentially dangerous cellular flotsam isn’t out of charac-



“Everyone thinks that OA [osteoarthritis] is simple wear and tear in the joint. ... Complement may play a crucial role in the breakdown of cartilage and destruction of the joint in OA.”

—WILLIAM ROBINSON,
STANFORD UNIVERSITY
SCHOOL OF MEDICINE



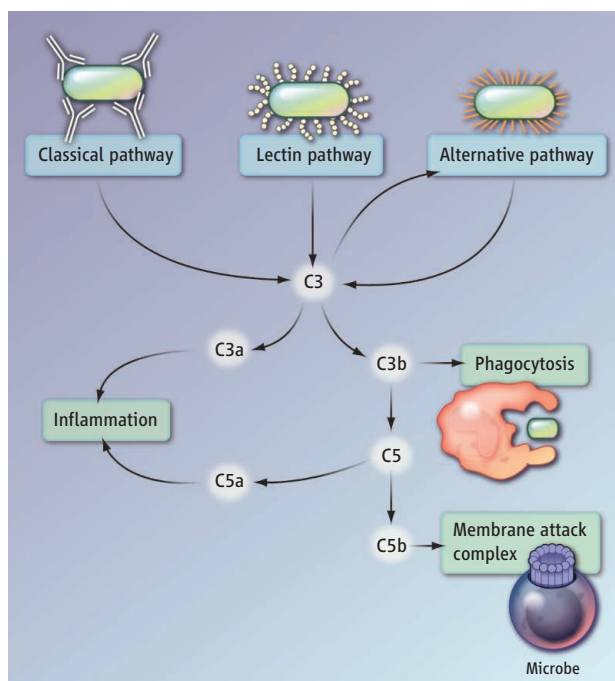
Breaking down. The knee of a control mouse shows more arthritic erosion (left, arrows) than one of a mouse that lacks the complement protein C5 (right).

ter. But recent revelations that complement helps steer normal development and fosters the repair and regeneration of damaged tissues have stretched our view of its contributions. Complement’s role in development is “the most striking” of its newly uncovered abilities, Sim says.

Several years ago, for example, a study led by neuroscientists Ben Barres of Stanford and Beth Stevens, now at Harvard Medical School in Boston, suggested that complement helps cut away unnecessary synapses during brain formation in young mice (*Science*, 14 December 2007, p. 1710). Earlier this year in *Neuron*, Stevens, Barres, and colleagues revealed how, showing that the complement protein C3 helps spur microglia, immune cells in the brain, to eat the unwanted connections.

Other studies point to developmental roles outside the nervous system. Last year, a multinational team of researchers reported that mutations in two complement genes were behind 3MC syndrome. Because children with this rare condition have facial deformities as well as learning disabilities, the finding indicates that complement helps shape the skeleton.

Another aspect of complement’s softer side is its role in the restoration of damaged tissues and organs. Unlike most other organs, the liver can regenerate after an injury. The



Lines of attack. This simplified diagram traces the main pathways of the complement cascade.

Stalling Sepsis?

Unlike osteoarthritis and age-related macular degeneration, sepsis is an illness in which you'd expect complement to be involved. Typically triggered by a bacterial infection that sends the immune system into overdrive, sepsis involves runaway, body-wide inflammation, with complement at the heart of the process. "We've found that C5a [a complement protein] plays a major role in sepsis," says immunopathologist Peter Ward of the University of Michigan Medical School in Ann Arbor.

More than 10 years ago, Ward and colleagues showed that dosing rodents with an antibody that sticks to C5a spares them from sepsis. In subsequent experiments in which they blocked the C5a receptors in mice and studied animals that lacked these molecules, Ward's team discovered how C5a makes trouble. In 2008 the researchers reported that C5a triggers a surge in the immune system signals known as cytokines, unleashing the so-called cytokine storm that can spur numerous organs in the body to stop working.

Several companies have begun testing C5a inhibitors for diseases such as atherosclerosis, and targeting the same molecule could be therapeutic for sepsis patients. Researchers are desperate for good news about the condition. More than 40 clinical trials of sepsis treatments have already failed, Ward notes, and the only drug approved in the United States specifically for sepsis, activated protein C, has been withdrawn from the market because of new evidence it doesn't work. Doctors can only offer general measures—such as broad-spectrum antibiotics and artificial respiration—that don't provide much benefit. In the United States, sepsis is fatal for almost 30% of the 750,000 people who fall victim to it each year. "It's a very frustrating situation right now," Ward says.

—M.L.

liver also manufactures most of the body's complement proteins—and the two capabilities seem to be related. "We have found that complement-deficient mice have impaired liver regeneration," Lambris says. An injury, such as one caused by a liver-damaging chemical, spurs production of the complement proteins C3a and C5a. The molecular details of how these proteins prompt the liver to refurbish itself remain unclear, Lambris says. But 3 years ago, he and his colleagues discovered that the proteins help keep dividing liver cells alive by activating a protective pathway.

The price of vigilance

Then there's the dark side of the complement system. "You need it, and if you don't have it you either get infections or you develop autoimmunity," Atkinson says. "But you don't want to turn it on a healthy cell."

Researchers are uncovering more and more instances in which that occurs. For example, complement attacks might take years off the working lives of transplanted organs. The cascade triggers much of the damage from so-called ischemia-reperfusion injuries, which occur after blood flow to a tissue or organ is temporarily cut off—such as by a blood clot or removal of the organ from a donor's body in preparation for transplantation. The two complement proteins C5a and C5b are the main culprits. C5a fires up damaging inflammation by stimulating immune cells known as neutrophils. Meanwhile, C5b and other proteins form membrane attack complexes that kill cells in the donor organ.

The effects of complement typically aren't severe enough to prevent a newly transplanted organ from working, says transplant immunologist Steven Sacks of the MRC Centre for Transplantation at King's College London. But all transplants eventually fail, and complement could hasten that process. "The question is why a 40-year-old organ doesn't last another 30 years," Sacks says.

He and his colleagues have developed a possible way to reduce complement-induced damage. The premise is that "the fate [of a transplanted organ] could be sealed based on the amount of reperfusion injury," Sacks says. So before implanting the organ, the research-



"The term 'complement' is a misnomer. It's an essential component of normal physiology and pathophysiology."

—JOHN LAMBRIS,
UNIVERSITY OF PENNSYLVANIA

ers wash it with an engineered artificial protein called mirococept, which sticks to cells in the organ and blocks all three branches of the complement system. The team has already completed a safety study of the compound in people, as well as a second study in 12 kidney transplant patients that produced encouraging preliminary data that the wash protected the donor organs from ischemia-reperfusion injury. Sacks says that a larger trial of mirococept will begin later this year at about 14 kidney transplant centers in the United Kingdom.

Hard on the eyes

Whether mirococept will prove itself in these trials remains to be seen, but some people with rare diseases are already benefiting from recent complement discoveries. The U.S. Food and Drug Administration has approved two anticomplement treatments. One is the antibody eculizumab, which latches onto the complement protein C5 and blocks the subsequent cascade. Doctors can now prescribe it for atypical hemolytic uremic syndrome, in which complement attacks the kidneys, and paroxysmal nocturnal hemoglobinuria, in which complement destroys blood cells. The second drug, Cinryze, blocks an enzyme in the complement cascade and ameliorates hereditary angioedema, in which out-of-control complement activity can cause symptoms such as swelling of the limbs and difficulty breathing.

Researchers predict that targeting complement will translate into other treatments. One disease that has already drawn a large amount of interest from scientists and drug companies is AMD. A combination of biochemical sleuthing and genome crunching connected complement to this macular degeneration, which usually strikes the eyes of people after age 50. In the disease, the portion of the retina that provides sharp vision deteriorates, often obliterating the central part of the visual field and leaving people unable to drive or read.

In the late 1990s, Gregory Hageman, now at the University of Utah School of Medicine in Salt Lake City; retinal cell biologist Don Anderson of the University of California, Santa Barbara; and colleagues decided to determine what was in the small globs of material called drusen that blemish the retinas of AMD patients. Thanks to Hageman, who

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was then at the University of Iowa in Iowa City, the researchers had access to plenty of eyes that had been donated to the university to provide corneal transplants; once the corneas were removed, the eyes were usually thrown away.

At first, the results of the analysis were puzzling, Anderson recalls. The initial protein the scientists identified in drusen was vitronectin, which, among other roles, naturally inhibits the activity of complement's membrane attack complex. Anderson says the team kept the findings under wraps for 2 years: "We were sitting around scratching our heads." But further probing of drusen revealed other complement proteins.

The case for complement's involvement in AMD grew stronger when researchers began checking for gene variants that were more common in patients with the eye disease. In 2005, four groups, including Hageman and Anderson's, reported that variants in the gene for factor H, a key complement inhibitor, boosted the risk of developing AMD. Researchers have since discovered that alterations in just three complement-related genes, including the one for factor H, account for about 75% of AMD cases in the developed world.

Before this work began, scientists ascribed AMD's retinal damage to factors such as smoking and high levels of blood lipids and "had no suspicions it was an immune disease," notes ophthalmologist and eye researcher Robyn Guymer of the University of Melbourne in Australia, who wasn't involved in the studies. "It really changed everyone's thinking about where to look in AMD." In a review published earlier this year, Guymer tallied the results of that change in perspective: At least eight complement inhibitors, including eculizumab, are undergoing preclinical or clinical testing for AMD.

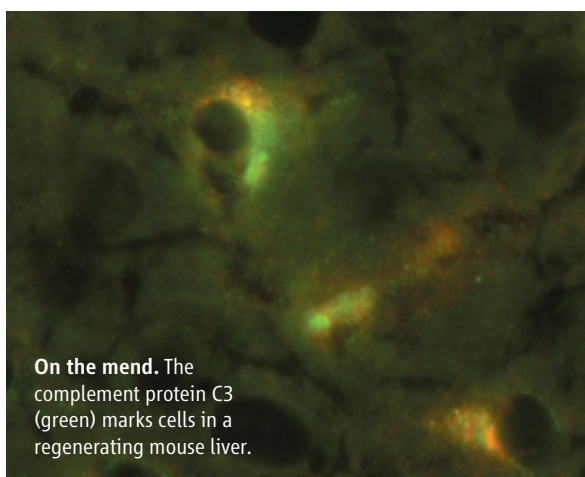
A drug problem?

Having two approved drugs for complement-related conditions is encouraging, researchers say. But both drugs have drawbacks, particularly their cost. A year's worth of eculizumab runs more than \$400,000, and Cinryze isn't much cheaper.

One possible route to more economical alternatives, Lambris says, involves small peptides that would be easier to manufacture. He and his colleagues have synthesized a molecule called compstatin that suppresses C3, the hub of the complement cascade. "We feel this is a good way to prevent complement

activation," says Lambris, whose university licensed the compound to a biotech company for further development. One benefit of interdicting the cascade at C3, he notes, is that it prevents complement from continually churning out compounds that switch on inflammation-promoting neutrophils. Lambris adds that several lines of evidence, including studies of other C3 inhibitors, suggest that this strategy is safe. Phase II trials, run by a second pharmaceutical company, are evaluating a modified version of the compound for AMD.

With more than 30 proteins, the complement system seems to offer plenty of targets for drug designers. But compstatin highlights one of the tricky questions in complement drug design: how to tamper with the cascade without subverting its antibacterial abilities. For example, some researchers worry that blocking C3 will prevent production of the key defender C3b, which spurs macrophages and other phagocytic cells to devour invaders. "If you inhibit complement early, ... you



On the mend. The complement protein C3 (green) marks cells in a regenerating mouse liver.

will seriously compromise innate immune function," says immunopathologist Peter Ward of the University of Michigan Medical School in Ann Arbor.

To limit possible side effects, some researchers favor concentrating on proteins further down the complement cascade. Eculizumab, for instance, targets the C5 protein. But Ward says he's concerned that even blocking C5 would leave people vulnerable to microbes; he notes that patients are required to get vaccinations against meningitis bacteria before receiving the antibody. Activated C5 splits into C5a, which ignites inflamma-



Changing places. Removing an organ for transplantation unleashes complement-mediated damage.

tion, and C5b, which joins the membrane attack complex that slays bacteria. A better alternative, Ward says, is inhibiting C5a. His lab has been investigating whether a C5a-disabling antibody is beneficial for sepsis in animals (see sidebar).

Immunologist Michael Holers of the University of Colorado, Denver, and colleagues have taken a different approach to minimize the collateral damage of interfering with complement. They devised a combo molecule that includes part of a complement receptor—a protein that enables our cells to respond to complement proteins—and part of the complement inhibitor factor H. The idea is that the drug, dubbed TT30, will home in on tissues where complement is active. The receptor portion of TT30 sticks to any of our cells that are under complement attack and allows the inhibitor to shield them from the onslaught, but the compound isn't a general immunosuppressant because it doesn't inhibit complement throughout the body. Now being developed by a pharmaceutical company, the drug has made it through Phase I safety trials, Holers says.

We might even find ideas for new complement therapies within our worst enemies, Lambris says. Human pathogens have fought an evolutionary battle against the complement system for hundreds of millions of years, and they've come up with some devious tricks to evade it. For example, *Staphylococcus* bacteria produce at least eight complement inhibitors that could serve as templates for new drugs, he says. Knowing our enemies better might help protect us from the friendly fire of one of our strongest defenses. —MITCH LESLIE

LETTERS

edited by Jennifer Sills

The Scientific Whaling Loophole

AT THE RECENT INTERNATIONAL WHALING COMMISSION'S ANNUAL meeting in Panama, South Korean officials announced their plan to initiate a "scientific whaling" program (1). This announcement came as a surprise given the general sentiment that the global demand for whale meat is declining. After weeks of international outcry, on 17 July, South Korea reversed their decision to hunt whales for research, but the issue is not dead (2).

South Korea claimed that the goal of the scientific whaling program is to study the types and amounts of fish whales eat, given conflict with fisheries. Yet, it is well established in the scientific literature that there are many ways to study whale diet without killing them (3).

Decades of fruitless negotiation between pro- and anti-whaling nations suggests a broken system, wrought with loopholes that allow unsustainable whaling to continue. Within this broken system, there is no incentive to reduce whaling, as the recent announcement by South Korea shows. Whaling groups are unwilling to compromise by allowing a sustainable

harvest of whales, so unsustainable (scientific) whaling continues.

To ensure a future of both whales and whalers, we must harness the passion and value that people place on living whales, without telling people what to do or force one set of values on others. We need novel, out-of-the-box approaches to effective management and conservation of whales. We must compromise to ensure reductions in whales being killed, better oversight of countries that harvest them, and limited whaling that does not threaten the persistence of whales.

For those who believe that whaling is unethical, I challenge you to put forward alternative ideas to a global moratorium that fosters the "loophole" of scientific whaling. With new plans to develop scientific whaling programs (4), the current global moratorium is clearly broken. Scientists, conservation advocates, resource managers, and the public must work together to develop new approaches to ensure the persistence of whales in our oceans.

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Iconic CO₂ Time Series
at Risk

THE STEADY RISE IN ATMOSPHERIC LONG-lived greenhouse gas concentrations is the main driver of contemporary climate change. The Mauna Loa CO₂ time series (1, 2), started by C. D. Keeling in 1958 and maintained today by the Scripps Institution of Oceanography and the Earth System Research Laboratory (ESRL) of NOAA, is iconic evidence of the effect of human-caused fossil fuel and land-use change emissions on the atmospheric increase of CO₂. The continuity of such records depends critically on having stable funding, which is challenging to maintain in the context of 3- to 4-year research grant funding cycles (3), and

is currently threatened by the financial crisis.

The ESRL Global Monitoring Division maintains a network of about 100 surface and aircraft sites worldwide at which whole air samples are collected approximately every week for analysis of CO₂, CH₄, CO, halocarbons, and many other chemical species (4). This is complemented by high-frequency measurements at the Mauna Loa, Barrow, American Samoa, and South Pole observatories, and about 10 North American tall towers. The success of the NOAA program has inspired similar efforts in Europe (5), China (6), India (7), and Brazil (8), with the United Nations World Meteorological Organization providing guidance and precision requirements through the Global Atmosphere Watch program (9), but no funding.

The data collected by NOAA and its worldwide partners have been used not only to demonstrate the unassailable rise of atmospheric greenhouse gas concentrations, but also to infer the magnitudes, locations, and times of surface-atmosphere exchange of those gases based on small concentration gradients between sites (10). Important findings from analysis of these records include the detection of a significant terrestrial carbon sink at northern mid-latitudes (11) and subsequent research aimed at identifying the mechanisms by which that sink must operate. Long-term, high-quality, atmospheric measurements are crucial for quantifying trends in greenhouse gas fluxes and attributing them to fossil fuel emissions, changes in land-use and management, or the response of natural

land and ocean ecosystems to climate change and elevated CO₂ concentrations.

Greenhouse gas measurements along tall towers in the interior continents allow quantification of regional sources and sinks, which has a very high relevance for measuring the effectiveness of climate policy. NOAA ESRL provides measurements that are critical for the U.S. national security in that they provide independent verification and early warning of changing greenhouse gas emissions from countries involved in efforts to mitigate greenhouse gases.

Dedicated carbon-observing satellites such as GOSAT and OCO-2 are needed to fill in the missing geographical information required for verification of carbon flux mitigation efforts. However, satellite retrievals do not yet provide sufficient information to deliver new constraints on surface fluxes, although quick progress is being made in this direction. In situ observations are crucial for anchoring space-borne measurements, for detecting potential biases of remote sensing techniques, and for providing continuity given the finite lifetime of satellites.

Despite the growing importance of greenhouse gas observations to humanity, sub-

stantial budget cuts at NOAA have resulted in curtailment of our ability to observe and understand changes to the global carbon cycle. Already, a dozen surface flask-sampling sites have been removed from NOAA's operational network and aircraft profiling sites have been eliminated and reduced in frequency at the remaining NOAA sites. The planned growth in the tall tower program has stopped, and plans for closing some towers are being developed. The U.S. budget process in this election year, with the added risk of mandatory across-the-board cuts due to the 2011 Budget Control Act, foretells more bleak news for greenhouse gas monitoring at NOAA and could cause further retreat from the goal of recording ongoing changes in atmospheric composition. As scientists, we believe that preserving the continuity of these vital time series must remain a priority for U.S. carbon cycle research.

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Many scientists and engineers contribute valuable time away from the established career paths of research, teaching, and publishing to foster activities and develop programs that both address key science questions and build important societal links. AAAS seeks to recognize an individual or a limited number of individuals working together in the scientific or engineering community for making an outstanding contribution to furthering science diplomacy.

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All materials must be received by September 1.



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LIFE SCIENCE TECHNOLOGIES

Proteomics

Panning the Proteome for Biomarker Gold



In This Issue

Targeted proteomics is homing in on promising biomarkers to help screen for cancer and guide patient treatment, but much work still needs to be done to validate these biomarkers and develop technology capable of bringing them to the clinic.

See full story on page 1120.

Upcoming Features

Genomics: Epigenetics/Epigenomics—October 26
Tissue Engineering: 3-D/Scaffolding—December 7
Connectome—January 18

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Letters to the Editor

Letters (~300 words) discuss material published in *Science* in the past 3 months or matters of general interest. Letters are not acknowledged upon receipt. Whether published in full or in part, Letters are subject to editing for clarity and space. Letters submitted, published, or posted elsewhere, in print or online, will be disqualified. To submit a Letter, go to www.submit2science.org.

Decoding Cryptosystems

R. STONE'S NEWS FOCUS STORY ABOUT PAN Jianwei's marvelous quantum optics experiments ("Entangled secret messages from space," 29 June, p. 1632) propagates some unfortunately common misconceptions about the uses of quantum key distribution (QKD) technology, especially its integration into a complete cryptosystem.

The confusion arises in the distinction between a cryptographic key and a communication session encrypted via the key. QKD does not carry or encrypt the message directly. Instead, QKD uses a classical communication authentication mechanism, quantum eavesdropping detection, and a healthy dose of statistics, as well as both quantum and classical randomness, to generate a random bit string that is known to be secret and shared only between two parties. This random bit string is then used as the encryption key for a standard, classical encryption system.

The ultimate success of the cryptosystem in protecting sensitive data depends on several factors. One such factor is the QKD implementation itself; no general attack against QKD is known, but various attacks have been proposed and even implemented against the photon generators, detectors, and electromechanical subsystems. Implementers respond by fixing problems in the usual thrust-and-parry of security systems implementation.

The security of the data depends on the strength of the classical encryption. The ideal use of the key would be to use it once and discard, as in a one-time pad (OTP), but current QKD key generation rates are far below desired classical communication rates, leading implementers to use the key for encryption schemes, such as Advanced Encryption Standard (AES), which encrypt many data bits with the use of fewer key bits. If used properly, OTP is perfectly secure, whereas AES could be broken by trying all possible keys, a theoretically possible but computationally impractical task.

Rather than flat statements that communication using QKD is totally unbreakable, it is more correct to say that it presents a different attack surface.

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CORRECTIONS AND CLARIFICATIONS

News Focus: "Where are the missing baryons?" by Y. Bhat-tacharjee (1 June, p. 1093). Oxygen VI is oxygen stripped of five electrons, not six, and Neon VIII is neon stripped of seven electrons, not eight.

This Week in Science: "Tic TOC1 plant clock" (6 April, p. 11). The editors note that the title of this summary was not intended to convey a connection between TOC1 and the plant gene *Tic*.

Reports: "The B73 maize genome: Complexity, diversity, and dynamics" by P. S. Schnable *et al.* (20 November 2009, p. 1112). Reference 27 should be C. Liang, L. Mao, D. Ware, L. Stein, *Genome Res.* **19**, 1912 (2009). The reference has been corrected in the HTML version online.

TECHNICAL COMMENT ABSTRACTS

Comment on "Intensifying Weathering and Land Use in Iron Age Central Africa"

K. Neumann, M. K. H. Eggert, R. Oslisly, B. Clist, T. Denham, P. de Maret, S. Ozainne, E. Hildebrand, K. Bostoen, U. Salzmann, D. Schwartz, B. Eichhorn, B. Tchiengué, A. Höhn Bayon *et al.* (Reports, 9 March 2012, p. 1219) interpreted unusually high aluminum-potassium ratio values in an Atlantic sediment core as indicating anthropogenic deforestation around 2500 years before the present (B.P.). We argue that there is no terrestrial evidence for forest destruction by humans and that the third millennium B.P. rainforest crisis can be clearly attributed mostly to climatic change.

Full text at www.sciencemag.org/cgi/content/full/337/6098/1040-c

Comment on "Intensifying Weathering and Land Use in Iron Age Central Africa"

Jean Maley, Pierre Giresse, Charles Doumenge, Charly Favier

Bayon *et al.* (Reports, 9 March 2012, p. 1219) claim that the "rainforest crisis" in Central Africa centered around 2500 years before the present "was not triggered by natural climatic factors" and that it was caused by widespread deforestation resulting from the arrival of the Bantu colonists. However, there is a consensus among palaeoecologists that this landscape change and the related physical erosion it caused was due mainly to a shift to more seasonal rainfall regime.

Full text at www.sciencemag.org/cgi/content/full/337/6098/1040-d

Response to Comments on "Intensifying Weathering and Land Use in Iron Age Central Africa"

Germain Bayon, Bernard Dennielou, Joël Etoubleau, Emmanuel Ponzevera, Samuel Toucanne, Sylvain Bermell

Neumann *et al.* argue that terrestrial evidence does not support our interpretation of large-scale human land use in Central Africa about 2500 years ago and that climate was the main driver of the rainforest crisis at that time, and Maley *et al.* raise a number of concerns about our interpretation of data from chemical weathering proxies. Taking into account existing palaeoclimatic data and clarifying some misconceptions, we reassert that humans must also have contributed fundamentally to this large vegetation change.

Full text at www.sciencemag.org/cgi/content/full/337/6098/1040-e

Comment on “Intensifying Weathering and Land Use in Iron Age Central Africa”

K. Neumann,^{1*} M. K. H. Eggert,² R. Oslisly,³ B. Clist,⁴ T. Denham,⁵ P. de Maret,⁶ S. Ozanne,⁷ E. Hildebrand,⁸ K. Bostoen,⁴ U. Salzmann,⁹ D. Schwartz,¹⁰ B. Eichhorn,¹ B. Tchiengué,¹¹ A. Höhn¹

Bayon *et al.* (Reports, 9 March 2012, p. 1219) interpreted unusually high aluminum-potassium ratio values in an Atlantic sediment core as indicating anthropogenic deforestation around 2500 years before the present (B.P.). We argue that there is no terrestrial evidence for forest destruction by humans and that the third millennium B.P. rainforest crisis can be clearly attributed mostly to climatic change.

Bayon *et al.* (1) reported geochemical results of a marine sediment record recovered in the Atlantic off the mouth of the Congo River. They interpreted aluminum-potassium ratio (Al/K) values in terms of weathering intensity in the Congo watershed and regional climatic developments. For most of the 20,000 years for which relevant data are presented, high Al/K values correlate with high rates of soil weathering during periods of increased precipitation, and low Al/K values correspond to low weathering rates under dryer conditions. The exception is a period of unusually high Al/K ratios in three samples, with inferred dates of 2444 to 2106 years before the present (yr B.P.), which led Bayon *et al.* to suggest that Bantu-speaking farmers were responsible for a major deforestation event during this period, with intensive land use and iron smelting resulting in soil denudation and increased weathering. The authors present interesting new data on the late Holocene palaeoenvironment of Central Africa. However, we strongly question their conclusion about the role of human impact

in deforestation during this period because their interpretation contradicts palaeoecological and archaeological evidence from terrestrial sites within, or adjacent to, the Congo drainage basin.

The hypothesis that a rainforest crisis resulting from climatic change in the third millennium B.P. facilitated the spread of agricultural communities throughout Central Africa (2, 3) has found broad acceptance by palaeoecologists and archaeologists. Numerous studies have modified the original model and suggest that a major vegetation change occurred in two phases. The first, around 4000 yr B.P., mainly affected the periphery of the central African forest block and can be attributed to decreasing rainfall. The second, caused by increasing seasonality of rainfall between 2500 and 2100 yr B.P., also was noticeable in the interior. The appearance of a marked dry season due to an abnormal southward shift of the intertropical convergence zone is corroborated by Saharan diatoms in dust deposited as far south as 4°N and the savanna crop *Pennisetum glaucum* in contemporaneous archaeological sites (4, 5). Although at the periphery savannas were spreading, the rainforest crisis of the third millennium B.P. was not a general “deforestation event,” as Bayon *et al.* argue. In southern Cameroon, Gabon and the inner Congo Basin, a mosaic of mature and secondary forests with light-demanding trees developed (4, 6). None of the palynological archives indicated in figure 1 of Bayon *et al.* shows any sign of human impact for this period; instead, rainforest disturbance can be readily attributed to increasing aridity and/or stronger seasonality.

Archaeological sites containing pottery, and in some cases also iron, with radiocarbon dates clustering in the second half of the third millennium B.P., attest to a major immigration into the central African rainforest. Insofar as the available calibrated dates (2 SD) allow a judgment, one might discern a slight tendency toward a north-to-south movement. However, many more dates are needed to affirm this. As yet, the oldest sites are located in southern Cameroon and

date to around 3000 yr B.P.; later ones occur in Gabon and on the coast of the Republic of Congo (Congo-Brazzaville) (around 2600 yr B.P.) and in the Democratic Republic of Congo (Congo-Kinshasa) (around 2400 yr B.P.) (7–9). The discrepancy between the timing of the localization of settlements and large-scale vegetation changes strongly suggests that human impact was not the major causal factor for forest disturbance.

Despite repeated claims, especially in the secondary literature (10), that the immigrants were farmers, direct data on their economies are scarce. Available evidence suggests a mixed subsistence system with hunting, fishing, collecting, animal husbandry, and, limited to southern Cameroon, some small-scale plant cultivation. People collected fruits and firewood near settlements and changed the species composition of the forest through some form of management. Archaeobotanical samples indicate a mosaic of mature and secondary forests, comprised of shade-tolerant and light-demanding trees, around the settlements (5, 7, 11). If agricultural plots were present, they must have been small. Common oil palm fruits do not prove intensified plant cultivation, as Bayon *et al.* state, but may have been collected from natural stands, as is done today. Pollen data clearly show that expansion of this pioneer species always followed climatically induced openings of the rainforest (4, 12). Linguistic, archaeological, and archaeobotanical data are consistent with the hypothesis that the settlers took advantage of the secondary forest plant communities, which can be easily cleared and contain numerous useful tree species.

Vegetation degradation due to metallurgy also is undocumented. The earliest evidence of iron production in the rainforest is dated around 2500 yr B.P., but such evidence is not voluminous. In Bas-Congo of the Democratic Republic of Congo and the Sangha region of the Republic of Congo, iron production appears even later, around 2000 yr B.P. (7, 8, 13), thus excluding any temporal correlation between increased Al/K values in the Atlantic around 2400 yr B.P. and potential deforestation for metallurgy on the continent. Even after the intensification of iron production in Central Africa after 2000 yr B.P., its influence on the vegetation remained negligible (14).

We do not deny that the first ceramic- and iron-producing immigrants had an impact on the rainforest and acted as potential amplifiers of environmental change. As in other parts of the world, the central African rainforest has been subject to human manipulation and is by no means “virgin.” Current debates regard tropical rainforests as cultural landscapes where prehistoric people developed special adaptations and management practices, thus influencing plant succession and species composition (15). So far, all available terrestrial evidence points to climate change as the major factor for vegetation transformation in the central African rainforest during the third millennium B.P., potentially facilitating

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the introduction of small-scale farming and iron production. Testing this hypothesis will require more firm data from archaeological sites, coupled with regional palaeoecological studies. Three geochemical samples from one marine core clearly are not sufficient to confirm large-scale anthropogenic forest destruction on the African continent as an alternative hypothesis.

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12 March 2012; accepted 4 June 2012

10.1126/science.1221747

Comment on “Intensifying Weathering and Land Use in Iron Age Central Africa”

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Bayon *et al.* (Reports, 9 March 2012, p. 1219) claim that the “rainforest crisis” in Central Africa centered around 2500 years before the present “was not triggered by natural climatic factors” and that it was caused by widespread deforestation resulting from the arrival of the Bantu colonists. However, there is a consensus among palaeoecologists that this landscape change and the related physical erosion it caused was due mainly to a shift to more seasonal rainfall regime.

Bayon *et al.* (1) analyzed the fluctuations in climate and vegetation in the Congo Basin during the last 20 millennia using aluminum-potassium ratios (Al/K) and hafnium isotopic composition of a marine sediment core retrieved off the mouth of the Congo River. They claim that the highest values of these ratios are evidence of intense chemical weathering during intervals that experienced the greatest amounts of annual precipitation, stressing that the degree of chemical weathering at about 3000 years before the present (yr B.P.) exceeded that of all other previous episodes. Bayon *et al.* recognize “a major vegetation change occurred in Central Africa, when rainforest trees were abruptly replaced by savannas.” However, a consensus exists among palaeoecologists, palynologists, and archaeologists that this event resulted mainly from a change in the seasonal regime of rains that did not involve a change in the annual amount of precipitation (2, 3). In our opinion, the increase in physical erosion that Bayon *et al.* observe was related to a large climatic change for two main reasons: first, because of the abrupt character of the event and, second, due to its very large geographical extension, from the Gulf of Guinea to the eastern end of the Congo basin and farther to the western part of East Africa (2), and throughout all northern tropical Africa from Cameroon (4) to Chad and West Africa (5, 6). Bayon *et al.* suggest that this event was linked to the arrival of the Bantu colonists in Central Africa, which would have immediately

produced large-scale deforestation due to agriculture and iron smelting. Moreover, Bayon *et al.* also confuse the issue by using the term “weathering” to qualify both the physical and chemical processes. Physical weathering must be specified every time as well as chemical weathering and, in the first case, terms such as “mechanical erosion” or “physical denudation” would be preferable. Furthermore, chemical weathering occurs first and enables the process of the physical weathering, but the processes generally are not linked immediately. A study of the sediment fluxes of big rivers, including the Congo, concluded that “an inverse correlation between weathering intensities and suspended sediment concentrations is observed showing that the regions having the highest rates of physical denudation produce the least weathered sediments” (7). Had there been increasing deforestation and physical erosion from the Bantu expansion, less chemical weathering, not more, should have occurred. Therefore, the Bantu expansion alone cannot explain the increases in this index seen by Bayon *et al.*

Assessing the chemical weathering ratio, the basis of Al/K, is an effective method in soil studies. This ratio rises when potassium minerals of the mica group (illites) are lost by hydrolysis. Then, potassium is solubilized and subtracted from the solid phase while residual aluminum is fixed, thanks to in situ kaolinite neoformation or gibbsite precipitation (Al(OH)₃) (8, 9). Measurements of Al/K usually are combined with mineralogical analyses such as the composition of clay minerals or the sandy fractions (with K-feldspar components), even if they are not abundant (4, 10). Bayon *et al.* supply no information about the textural or mineralogical nature of the marine sediments, which severely limits the relevance of their conclusions. Last, to use this method to assess marine sediments means implicitly assuming that a hypothetical process on the continent would be translated

almost immediately into riverine fluxes and then into oceanic deposition, which is hardly believable. Bayon *et al.*’s observation that “Hf isotopes display significant downcore variations... which correlate well with the Al/K depth profile” could simply mean that, repeatedly, erosion was able to affect deeper horizons of the soils where the signatures of chemical weathering may have been more or less intense and more or less ancient.

Therefore, the assertion that “these data show that downcore fluctuations of both ϵ_{Hf} and Al/K ratios... reflect variations in chemical weathering intensity within the Congo” is not very convincing because the erosion of modern superficial horizons of soils can interfere with that of the oldest horizons, so only strong chemical weathering could be apparent. The clear conclusion that emerges from this comparison (i.e., of big rivers of the world, including Congo) is that the degree of weathering of present-day river particulates is not consistent with most river dissolved loads derived from silicate weathering. This apparent disagreement is due to the fact that river suspended sediments integrate the whole weathering history of the rocks (recycling) within the drainage basin and not only the present-day weathering (4). The low values of ϵ_{Hf} at 20,000 yr B.P. (dry climate and many grasses) and its small decrease, for the same reasons, during the Younger Dryas, seem coherent, contrary to the strong increase by 2500 yr B.P. in a landscape more open than in the previous period and with more irregular rains. Apart from a possible hypothesis of a massive colonization by the Bantus, the other logical explanation of such data would be the erosion of fossil soils horizons, evidence of the leaching during past climates.

If the authors’ statement that “chemical alteration in the Congo Basin [which is confused here with Central Africa *sensu lato*] has responded quickly to regional climatic changes” is acceptable on the scale of 10³ years, it is not acceptable on the much shorter scale of 10² years, which is that of the presumed catastrophic event of Bantu colonization. Therefore, the assertion that “an abrupt trend toward higher Al/K and Hf isotope values indicates rapidly intensifying chemical weathering... centered at ~2500 years B.P.” seems to us exaggerated, especially when this episode is proposed to be the major weathering signal during the past 40,000 years. Moreover, the curves in Bayon *et al.* show a decrease of “weathering intensity” after 2000 yr B.P., whereas archaeological records—based on agricultural practices, iron-smelting industry, and ceramics—indicate that the very beginning of the Bantu colonization in the Congo Basin occurred about 2000 yr B.P. and reached a maximum only around 1000 yr B.P. (11, 12, 13). This is in complete contradiction of the suggestion of Bayon *et al.* that Bantu expansion was the main driver of this increase in weathering.

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12 March 2012; accepted 4 June 2012
 10.1126/science.1221820

Response to Comments on “Intensifying Weathering and Land Use in Iron Age Central Africa”

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Samuel Toucanne, Sylvain Bermell

Neumann *et al.* argue that terrestrial evidence does not support our interpretation of large-scale human land use in Central Africa about 2500 years ago and that climate was the main driver of the rainforest crisis at that time, and Maley *et al.* raise a number of concerns about our interpretation of data from chemical weathering proxies. Taking into account existing palaeoclimatic data and clarifying some misconceptions, we reassert that humans must also have contributed fundamentally to this large vegetation change.

In our recent study (1), we used aluminum-potassium ratios (Al/K) and other geochemical tracers in clays deposited off the Congo River to reconstruct past changes of chemical weathering intensity in Central Africa for the past 40,000 years. For much of that time, sedimentary Al/K ratios covaried well with the long-term climatic signal of the Late Quaternary period, indicating that chemical weathering processes in central African soils had intensified during humid periods, and vice versa. However, after about 3000 years ago, the Al/K profile departed significantly from the climatic signal toward unexpectedly high ratios indicative of more pronounced chemical weathering. Because this period coincided with both a major vegetation change and the arrival of the first farmers in Central Africa, we concluded that this weathering episode could reflect the effect of early agriculture on the rainforest. Our conclusions have been challenged by Neumann *et al.* (2) and by Maley *et al.* (3).

In their comment, Neumann *et al.* (2) disagree with our interpretation, arguing that there is no terrestrial evidence for massive forest destruction by humans at that time and that climate was the primary factor responsible for the vegetation change. Neumann *et al.* provide a detailed and interesting review of the way of life of the Bantu-speaking peoples who immigrated into the central African rainforest about 3000 years ago. As pointed out by the authors, available archaeological data suggest that those early farmers relied on a mixed subsistence system, involving arboriculture and small-scale plant cultivation, mainly taking advantage of secondary forest plant communities, which they could easily clear with presumably minor impact on the environment. Neumann *et al.* also indicate that iron metallurgy, which

developed in Central Africa after ~2500 years before the present (yr B.P.), is unlikely to have had major influence on the vegetation. Finally, the authors relate the major vegetation change recorded in terrestrial records at that time to an abnormal southward shift of the rainbelt, leading to the onset of marked dry seasons.

Since the end of the African Humid Period, about 6000 years ago, African climate has evolved progressively toward dryer conditions in response to reduced summer insolation and ocean circulation changes (4, 5). Superimposed on this gradual drying trend, several centennial- to millennial-scale episodes of low precipitation also occurred during that period. Although the onset of those minor climatic deteriorations is apparent in a few palaeoclimatic archives (5, 6), their impact on the rainforest remains unclear.

At present, vegetation patterns in Central Africa match annual precipitation rates remarkably well (7). Although the controls on past vegetation are still under debate, the extent of rainforest trees versus savannas (and other secondary formations) in Central Africa during the Late Quaternary correlates well with sea surface temperature (SST) in the eastern equatorial Atlantic. Tropical SST, in turn, is thought to control continental precipitation in this region (8–10). The large vegetation changes that occurred over interglacial/glacial time scales corresponded to SST changes of about 3° to 5°C in the tropical Atlantic (8, 9). A number of minor millennial-scale SST oscillations (about 1°C or less) also took place in the Gulf of Guinea during the Holocene period, including one between about 4000 and 2500 years ago (11). These small SST drops were related to periods of lower lake levels and reduced moisture availability in Central Africa (11). Most likely, these minor climatic deteriorations, superimposed on the gradually drying trend of the late Holocene African climate, also had some effect on the rainforest.

The rainforest crisis that took place in Central Africa between 3000 and 2000 years ago is much greater, however, than what would be expected

from the small SST variations recorded in sediment records from the Gulf of Guinea. At some sites (12), the disturbance inferred from palynological records for the third millennium B.P. is even comparable in magnitude to the major vegetation changes that accompanied the last deglaciation period. At first glance, this apparent discrepancy may seem hard to reconcile with a “simple” climatic hypothesis and could indicate that another factor came into play. However, as mentioned by Neumann *et al.*, the scarce archaeological data available do not support large-scale human impact at that time, which could possibly account for the rainforest crisis. In addition, palaeo-environmental data acquired from scattered terrestrial records do not generally allow clear distinction between climate-induced vegetation changes and anthropogenic disturbances.

Our chemical weathering proxy records overcome some of those difficulties. First, in contrast with vegetation-based proxies, weathering tracers respond in opposite directions to reduced precipitation levels (which induce lower chemical weathering intensity) and increasing human activities (which lead to both enhanced soil denudation and chemical weathering). This makes them particularly well suited for discriminating between climate- versus human-driven changes in past environmental records. Second, our marine sediment core (KZAI-01) provides an integrated record of Late Quaternary environmental conditions at the scale of the whole Congo Basin, something that is not attainable with terrestrial records. We estimate that the achievable temporal resolution in core KZAI-01, inferred from the combined effects of sediment transfer time from continent to ocean, bioturbation, and sedimentation rates, is about 600 years. To some extent, this relatively low temporal resolution (compared with terrestrial records) probably accounts for the smooth geochemical profiles in our core (1), possibly explaining why the minor millennial-scale events, which punctuated the central African climate during the Late Quaternary (e.g., at ~8.2 kyr and 4 kyr B.P.), were not recorded at site KZAI-01. In this context, the sudden pulse of intensifying chemical weathering inferred from our sedimentary record after 3000 yr B.P., and its complete decoupling from the long-term weathering signal of the past 40,000 years, is also hard to reconcile with a “simple” climatic hypothesis.

So why did weathering rapidly intensify at that time, when it had previously always decreased during dry episodes? We believe that the answer is related to the introduction of agriculture by the Bantu-speaking immigrants.

There is increasing evidence that humans have strongly altered their landscapes since the advent of agriculture (13–15). In every part of the world, diffusion of agriculture led to rapidly growing population, with increasing effects on the environment. Early farmers practiced slash-and-burn cultivation for clearing plots for agriculture. Even if they abandoned or rotated among pre-

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viously farmed plots, the Bantu-speaking agriculturalists would have left behind a lingering footprint on the land that was not reforested for several decades or centuries, thereby being subject to intense soil denudation and chemical weathering. Current estimates actually suggest that humans became the dominant agents of soil erosion (over natural causes) as early as the third millennium B.P. (16). Based on the above considerations, therefore, and although we fully understand that clear archaeological evidence may still be lacking, we reiterate our conclusion that the early farmers who immigrated into the central African rainforest about 3000 years ago already had a substantial impact on their environment.

Maley *et al.* (3) criticize our interpretations of Al/K data, raising a number of concerns about their reliability for tracing past changes in chemical weathering intensity. They also disagree with our conclusions and argue that the third millennium B.P. crisis of the central African rainforest was mostly the result of climate change, specifically due to a change in the seasonal regime of rainfall. Below, we address each of the issues raised by the authors about the interpretation of our data. Our views on the “climatic” hypothesis proposed by the authors are expressed above in the response to Neumann *et al.*

Maley *et al.* point out that the absence of mineralogical data for the detrital phases analyzed in our study strongly limits the relevance of our conclusions. This seems largely overstated, because it is well known that K is predominantly contributed by feldspars in Congo River-borne sediments, and Al by both feldspars and kaolinite (17).

Maley *et al.* also raise important concerns about possible time lags between soil erosion, river transport, and deposition of sediments on the ocean floor, and question whether chemical weathering processes could react as quickly as we suggest to past environmental changes. Based on these considerations, Maley *et al.* argue that the elevated Al/K ratios determined in our core after 3000 yr B.P. could reflect the erosion of deeper (more ancient) soil horizons, rather than any contemporaneous weathering signal.

Although the global relationship between chemical weathering and environmental parameters such as temperature and precipitation is well established, there are still large uncertainties on the sensitivity of weathering rates and intensities to these climatic variables, mostly because results obtained from laboratory experiments are difficult to reconcile with natural field-based approaches (18). Clearly, the time over which natural chemical weathering occurs cannot be reproduced by experimental studies, so the strongest evidence that chemical weathering in Central Africa reacted quite rapidly (within about 500 years) to past environmental changes actually comes from the application of various weathering proxies to eastern Atlantic sedimentary records. In addition to our work, several investigations based on clay mineralogy or major-element geochemistry have

already suggested that past variations in chemical weathering intensity in the Congo Basin were tightly coupled with climate change (17, 19).

There is also considerable uncertainty about how much time is needed for sediment in a river basin to reach the ocean. The residence time of sediments on continents can vary from a few years to many thousands of years, although clays are expected to be exported much more rapidly than coarser fractions. In our paper (1), we addressed this important issue by dating the organic fraction of several bulk sediment samples, in addition to the marine carbonate material used for establishing the age model for core KZAI-01. Because marine sediments deposited off the Congo River contain a significant fraction of continental organic matter (20), radiocarbon dating of bulk organic compounds can be used to calculate the age of river-borne particles at any sediment depth and hence to provide an estimate for past sediment transport times within the drainage basin. In our study, the transport times inferred from those radiocarbon dates were generally less than 600 years (1). This provided reassuring evidence that the suspended particles delivered by the Congo River during the last few millennia were mainly derived from relatively young soils and hence that the elevated Al/K ratios found after 3000 years ago were not derived from the erosion of older tropical soils.

Another, perhaps stronger, line of evidence against the possible contribution of fossil soils to our Al/K signal comes from results obtained during soil investigations. Typical weathering sequences in soil/regolith profiles from tropical environments show that Al/K ratios generally decrease gradually from the topsoil layers to the deeper lateritic horizons (21, 22), a consequence of progressive K depletion by leaching and accumulation of Al in secondary weathering products. Importantly, this suggests that erosion of deep (ancient) soil horizons in the Congo Basin would have led to export of clays characterized by lower Al/K ratios, which would be incompatible with the trend shown by our data.

Maley *et al.* also argue that increasing human activities during the third millennium B.P. should have led to less intense chemical weathering in soils (and hence to clays having lower Al/K), rather than, as we suggested, enhanced weathering and higher Al/K. This claim is based on results obtained during a geochemical survey of suspended sediments from the world's largest rivers (23). In that study, the proposed relationship between high physical denudation rates and low chemical weathering intensities was established to account for the large differences observed between rivers draining a wide range of geologic, climatic, and tectonic settings. Lowland tropical and soil-mantled regions, such as the Congo Basin, are indeed characterized by low denudation rates and intense weathering processes, whereas mountainous areas and volcanic islands exhibit the highest rates of mechanical erosion but produce the least-weathered sedi-

ments (because thin soil covers in these regions generally prevent intense mineral-water reactions to occur). However, at the scale of individual drainage basins, this relationship is largely obscured by the effects of other factors, such as regional precipitation, temperature, and/or vegetation.

Finally, Maley *et al.* comment on a presumed discrepancy between the timing of our Al/K weathering episode (centered around 2500 years ago) and subsequent intensification of human activities in Central Africa. Although the rise of Al/K ratios in core KZAI-01, initiated about 3000 years ago, coincided well with the arrival of the first Bantu-speaking farmers in Central Africa (2), we agree with Maley *et al.* that the Al/K decrease after 2000 yr B.P. may seem, at first glance, rather peculiar. One possible explanation for this trend actually also involves humans. Current archaeological evidence suggests that human populations in the Congo Basin crashed relatively abruptly ~1500 years ago, resulting in widespread forest regeneration (24, 25). The observed hiatus lasted for about six centuries, before human activities started again after ~1200 C.E. If correct, these observations would be coherent with the observed decrease of Al/K ratios at site KZAI-01 between ~2000 and 1400 years ago (i.e., the sediment age for the core top), which implies that forest regrowth at that time led to a decrease of soil erosion rates and chemical weathering processes in soils. Therefore, we are still confident that the Al/K profile for core KZAI-01 represents a reliable indicator of past chemical weathering in Central Africa and that its deviation from the long-term climatic signal after 3000 yr B.P. reflects the rapid development of agriculture at that time.

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Acknowledgments: This work was sponsored by the French National Research Agency (ANR) via the ECO-MIST project (2010 JCJC 609 01).

27 April 2012; accepted 4 June 2012
10.1126/science.1222458

EVOLUTION

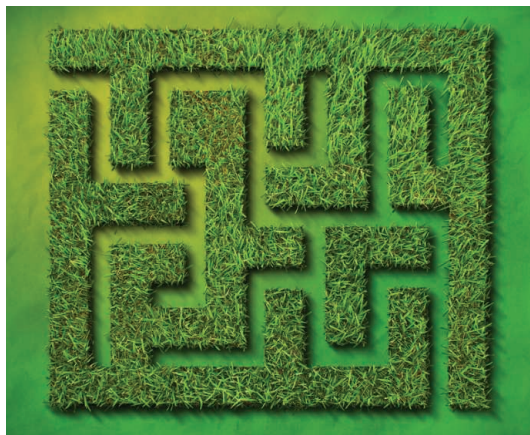
A Creation Story for Humanity

Rudolf Griss

Edward O. Wilson is not afraid to ask big questions—questions that religions, the creative arts, and philosophy have wrestled with for centuries. What is it that makes humans what they are? How did our human condition develop? How did nature give rise to something so unusual as ourselves—a species that feels empathy and guilt, cares for the old and sick, and tries to intellectually understand itself and its origins—with our languages, religions, arts, and cultures? With *The Social Conquest of Earth*, Wilson endeavors to uncover the creation story of humanity.

Generally speaking, animals are selfish. Evolution disfavors genes that promote altruism while reducing an individual's own fitness. Yet, there are ants and bees in whose colonies workers sacrifice their chance to have offspring in order to serve the queen. There are humans who risk their lives to save others—whether millennia ago on the hunt or today at war. Kin selection theory has been generally accepted as the best explanation for such counterintuitive behavior in these and other species. Close relatives share a large part of their DNA; genes of an individual can therefore be passed on to the next generation not only directly but also indirectly through a relative. The spread and survival of a gene may be better achieved if it leads its host to help a relative, so long as the benefits for the relative outweigh the costs for the host as well as the risk that this relative does not share the gene after all.

In 2010, Wilson—a renowned evolutionary biologist, ecologist, and student of social insects—and colleagues stirred up the field (1) and sparked a heated debate (2–7) by claiming that the firmly established theory of kin selection was flawed. Instead, they argued, group selection is the key to solving the riddle of altruism. Regardless of relatedness, genes can be favored by evolution even if they are not of advantage to an individual itself so long as they provide an advantage to a group to which the indi-



Evolutionary pathway. Wilson suggests visualizing the evolution of a species as a journey through a maze presented by the environment, a maze that can itself change with time.

vidual belongs. To simplify, in the case of two competing groups, cooperation among members of one can give it an advantage over the other. So it will be favored by natural selection—along with its members and the genes prescribing the cooperation.

Multilevel selection—the interplay between individual and group selection—provides the foundation for most of Wilson's explorations in the book. For example, he holds that it created the human dilemma of good and evil: Individual selection is responsible for the evil; within a group, selfish individuals win. But their success is countered by group selection, which is responsible for the good; a group of cooperating individuals wins against a group with selfish members. Humans are thus pulled toward selfishness by individual selection and toward altruism by group selection—destined to always be torn between the two sides.

Seeing *The Social Conquest of Earth* as simply one side of a scientific argument would greatly underestimate the book's value. Regardless of whether Wilson's theories lead to a paradigm shift in evolutionary biology—and even if one disagrees with various of his conclusions—the book certainly accomplishes one thing: it gets the reader thinking. Why do we follow religions or sports teams? Why are we racist? Why

do we go to war? Why do we feel empathy and honor? Why do we prefer to live close to rivers or lakes and enjoy having a view? Wilson tries to answer these and many other questions. He intersperses his discussions of human genetic and cultural evolution with stories of the astonishing evolutionary achievements of the social insects (e.g., ants holding sap-sucking insects as dairy cows or growing fungus in air-conditioned farms).

The author further enlivens the book with personal anecdotes. In a New Guinea rainforest, he tastes the excrement of scale insects and finds it “mildly sweet.” In “among the most exciting moments” of his life, he holds a 90-million-year-old piece of metasequoia amber that contains two beautifully preserved fossil ants (almost twice as old as any known previously)—and drops the amber, breaking it into two fragments (but, luckily, leaving an undamaged specimen in each).

After many stimulating insights and “aha” moments, one cannot avoid wondering how far self-understanding can go. Will we ever fully fathom the human mind when it is the only tool at our disposal to do so? But achieving such comprehension may not be the author's primary goal. As intriguing as self-understanding may be from an intellectual point of view, Wilson sees it as a means to an end: something that must be achieved if humans are to bring their unsustainable and destructive lifestyle to a halt. He laments, “We are needlessly turning the gold we inherited from our forebears into straw, and for that we will be despised by our descendants.” We are not only polluting our planet beyond recognition, we are also bidding good-bye forever to species after species. Wilson tells us to grow up, to stop making excuses and shuffling off responsibility onto deities. We alone are responsible for the future of our planet. The sooner we understand who we are and where we come from, the sooner we will know where we need to go.

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10.1126/science.1225640

The Social Conquest of Earth

by Edward O. Wilson

Liveright (Norton), New York, 2012. 341 pp. \$27.95, C\$29.50, £18.99. ISBN 9780871404138.

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ANTHROPOLOGY

The Source of Our Morality

Buyun Zhao

Prima facie, morality (our sense of right and wrong) appears to be an evolutionary paradox. Behaviors such as extrafamilial generosity are susceptible to exploitation, which naturally leads us to wonder what driving force has sustained them. In *Moral Origins: The Evolution of Virtue, Altruism, and Shame*, social anthropologist Christopher Boehm delves into his own research to offer a hypothesis for the conception of our moral sense. With its cautious rhetoric and deep introspection, his account provides a convincing tale.

Taking advantage of the potential for elaboration that a book provides, Boehm (University of Southern California) works back from hunter-gather populations to our primate relatives and contextualizes our moral beginnings with the natural history of our evolutionary lineage. To summarize his complex story succinctly: Erratic environmental shifts during the Pleistocene compelled individuals to collaborate in larger egalitarian units for more efficient hunting. Endowed with a nascent sense of equality, the hunting groups punished or shunned social renegades. Through such enforcement, “the social preferences of group members and of groups as a whole” had systematic effects on gene pools. Selection favored individuals “who were better at inhibiting their own anti-social tendencies, either through fear of punishment or through absorbing and identifying with their group’s rules.” Thus it helped stabilize egalitarian rule and keep freeloaders and bullies in check.

Uncomfortably inherent in this account, the counterintuitive notion that our sense of fairness arose prior to the formation of our conscience presents us with a philosophical dilemma. However, Boehm tactfully



Origins story. Bas-relief (14th century) on a pier of the facade of the cathedral, Orvieto, Italy.

Moral Origins

The Evolution of Virtue, Altruism, and Shame

by Christopher Boehm

Basic Books, New York, 2012. 426 pp. \$28.99, C\$33.50, £18.99. ISBN 9780465020485.

argues that understanding the rules of the social game should precede its true emotional internalization. He suggests that our conscience arose merely as a “Machiavellian risk calculator”—a process of thoughts that conceptualizes the game theory of prohibitive punishment costs versus defection benefits. This seems to me the most persuasive description of the emergence of conscience yet.

At times the book becomes frustratingly repetitious, but Boehm’s candid enthusiasm nearly always compensates for the ennui of reiteration. Nonetheless, in places his case might have been strengthened with alternative or additional examples. One that he often (and perhaps dangerously) relies on is Colin Turnbull’s account of how Mbuti pygmies deal with social offense (*I*). In this scenario, a member of an egalitarian pack oversteps the agreed hunting strategy to gain more game. Through gossiping among his peers, he is faced with holistic social ostracism, the resolution of which involves the confiscation of his meat. Although this anecdote might help explain how early tribes elicited social selection, gossiping is a double-edged sword. Especially during difficult times, the incentive to use malicious lies as a means of getting a slightly larger share of the food could become considerable; what’s more, catching a liar is no easy task.

Toward the conclusion of the book, Boehm’s narrative enters a curious turbulence in which he explains his optimism for the progress of neuroscience and for our future. This consideration of “humanity’s moral

future” seems to undermine the cohesion of his account as well as the author’s authority. Venturing into the topic of our world of nations, he precariously draws comparisons between the egalitarian order of hunter-gatherers past and international dynamics of the present and future. Noting that “like a foraging band our global community of nations is vehemently unwilling to trust a centralized system of command and control,” Boehm finds hope in the “degree of insight and realistic goodwill exhibited by our Pleistocene hunting forbears, when they realized that if they wanted to live well as hunters they had to merge their competing interests and cooperate, and they proceeded to do

just that in the absence of strong chiefs.” He also sees grounds for optimism in “our evolutionary gifts of sympathy and generosity.” Simply to be provocative, I offer an alternative view: Given that we live in a world where the strong initial selection pressures (and penalties) of morality are absent, it might be reasonable to conclude that we’ve passed our genetic moral peak and hence our predisposition as moral beings is waning.

Skimming *Moral Origins*, it would be easy to misplace the insights that Boehm offers—after all, the notion of moralistic social selection has been entertained since Plato’s *Republic*. The book’s greatest value lies in its elegant naturalistic explanation for morality, which dovetails Darwinian history with philosophy. Walking a fine line between loquacious charm and critical finesse, Boehm provides a stimulating account infused with probing thought experiments and open questions. Although he makes no pretense that it will be the final word on the development of morality, his evolutionary scenario is profoundly satisfying. Boehm’s conclusion is not the last missing piece of the puzzle, but it may be one of several that we need for a holistic understanding of the origins of our morality.

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10.1126/science.1225641

CLIMATE CHANGE

Climate Negotiators Create an Opportunity for Scholars

Joseph E. Aldy and Robert N. Stavins*

The 1992 United Nations Framework Convention on Climate Change (UNFCCC) launched a process to confront risks posed by global climate change. It has led to a dichotomy between countries with serious emission-reduction responsibilities and others with no responsibilities whatsoever. This has prevented progress, but recent talks suggest the prospect for a better way forward and an openness to outside-the-box thinking. Scholars and practitioners have a new opportunity to contribute innovative proposals for a future international climate policy architecture.

Beyond a Dichotomous Distinction

Article 3 of the 1992 UNFCCC established a key principle: “The Parties should protect the climate system ... on the basis of equity and in accordance with their common but differentiated responsibilities and respective capabilities. Accordingly, the developed country Parties should take the lead ...” (1). As a result, the UNFCCC explicitly recognized that developed countries should “take immediate action” as “a first step towards comprehensive strategies” to address climate change (2). This differentiation in action dates back, in some form, at least to the 1972 Stockholm Declaration on the Human Environment and was incorporated in the 1992 Rio Declaration on Environment and Development.

In the first decision of the first Conference of the Parties (COP-1) of the UNFCCC, the global community agreed to the Berlin Mandate, which interpreted “common but differentiated responsibilities” in which “developed country Parties” (also known as Annex I countries) alone are to take on emission-reduction responsibilities. The Berlin Mandate, codified with numerical national targets and timetables for Annex I countries in the 1997 Kyoto Protocol, produced a dramatic gap between rhetoric and reality. By the time of the Berlin Mandate, non-Annex I countries’ annual greenhouse gas emissions



Negotiating political architecture. Both developed and developing countries need to be engaged in emission reduction initiatives.

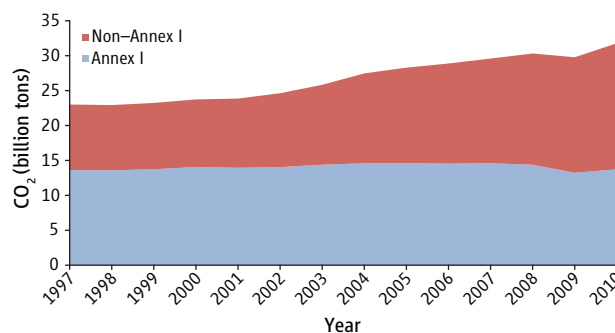
surpassed those of Annex I countries (3). By 2005, when the Kyoto Protocol entered into force, per capita fossil fuel carbon dioxide emissions of nearly 50 non-Annex I countries exceeded those of the Annex I country with the lowest per capita measure (4) (see the chart). Further, the six largest greenhouse gas emitters are not constrained by the Kyoto Protocol, because of lack of commitments (China, Indonesia, Brazil, and India), the nonbinding nature of its emission commitment (Russia), or failure to ratify the agreement (United States).

The dichotomous structure effectively quadruples the global cost of emission abatement necessary to stabilize atmospheric con-

centrations of greenhouse gases relative to a cost-minimizing scenario that includes emission abatement by all nations (5). The Kyoto Protocol provides no means for developing countries to take on emission targets and engage in international emission trading, because some of the largest developing countries actively opposed a voluntary accession mechanism at the 1997 Kyoto negotiations. Argentina offered to take on an emission target in 1999 but could not even secure agreement to have this discussed on the negotiating agenda. Thus, the Kyoto Protocol severely limited opportunities for developed countries to leverage finance of low-cost emission abatement in developing

countries (e.g., domestic cap-and-trade, fossil fuel subsidy reform, and building codes) through international emission trading under emission targets.

But prospects for change emerged in 2009. Leaders of the seventeen largest developed and developing countries, at the Major Economies Forum on Energy and Climate, agreed that they would need to reduce their greenhouse gas emissions. Leaders of these economies and many more nations negotiated the Copenhagen Accord later in 2009, fol-



Annex I and non-Annex I fossil fuel carbon dioxide emissions, 1997–2010. Since the 1997 Kyoto Conference, Annex I countries’ emissions of fossil fuel-based carbon dioxide have remained level while non-Annex I countries’ emissions have nearly doubled and stood nearly one-third greater than developed countries’ emissions in 2010. This gap is likely wider when accounting for all greenhouse gas emissions. Data from U.S. Energy Information Administration (4). See supplementary materials for details.

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lowed a year later by the Cancun Agreements (December 2010), which together blurred the distinction between Annex I and non-Annex I. Under Copenhagen and Cancun, developed countries pledged economy-wide emission targets, and nearly 50 developing countries pledged emission mitigation policies and actions.

An even greater departure from the Annex I/non-Annex I dichotomy took place at the most recent negotiations in Durban, South Africa, in December, 2011. At the COP-17 talks in Durban, the international community agreed to a negotiating process focused on long-term participation of all parties in the effort to mitigate greenhouse gas emissions (6). The Durban Platform for Enhanced Action (DPEA) calls for a comprehensive legal regime by 2020 that essentially eliminates the Annex I versus non-Annex I distinction.

We ought not to overestimate the importance of a nonbinding agreement to reach a future agreement, especially as some developing countries have considered stepping back from this agreement. Nonetheless, this is a significant departure from the past. It is of vast potential importance, but only “potential,” because just as the Kyoto Protocol’s targets and timetables fulfilled the Berlin Mandate’s promise, future COPs must deliver on the DPEA with a new post-Kyoto agreement by 2015.

Architectural Evolution

Many international policy architectures exist that could be consistent with the process and principles laid out in the DPEA and the UNFCCC. A top-down formulaic approach to reforming Kyoto could set 5-year emission targets for all countries through 2100 based on four equity principles (7): “progressivity,” adjusting emissions targets based on per capita income; “latecomer catch-up,” helping close the gap between 1990 emissions (the Kyoto baseline) and the starting points for latecomers (e.g., Canada, China, and the United States); “equalization,” aligning targets with global average per capita emissions by the end of this century (8); and an economic feasibility constraint that costs should not exceed a particular share of Gross Domestic Product.

Emissions targets for major developing countries could be set at “business-as-usual” (BAU) emissions levels but become more stringent as countries become wealthier. Keeping poor countries near BAU emissions prevents carbon leakage, an increase in emissions in one country resulting from a decrease in another country. Combining BAU

targets with an international emission-trading program could provide direct economic incentive (export revenues and foreign direct investment) for developing-country participation. By taking on BAU targets, developing countries could create a new export industry by reducing emissions below BAU and exporting emission allowances to developed countries. Developing countries could fully participate without incurring prohibitive costs, addressing cost effectiveness and distributional equity concerns.

Greenhouse gas cap-and-trade systems are in place or under development in the European Union, Australia, Japan, Korea, New Zealand, California (USA), and several Canadian provinces; a global emission reduction credit scheme, the Clean Development Mechanism, has supporters in developing countries. There is interest in linking cap-and-trade systems to allow the use of allowances or credits across systems to meet compliance obligations. Linking increases liquidity and functioning of markets and can greatly reduce abatement costs (9). However, direct linkage of cap-and-trade systems will propagate certain cost-containment design elements from one system to another, so advanced harmonization could be required, akin to bilateral trade agreements that were a precursor to today’s multilateral trade regime.

However, when cap-and-trade systems link with a common emission reduction-credit system (which does not have a cap, but issues credits when emissions are reduced below some agreed baseline), indirect links among cap-and-trade systems are created but without propagation of those design elements of concern. Such indirect linkages can thus achieve many of the benefits that direct links achieve but without the need for advanced harmonization. Bottom-up, indirect international linkage has begun to emerge, and may be part of the de facto international climate-policy architecture.

Finally, the UNFCCC regime for measurement, reporting, and verification is inadequate. For example, China’s most recent greenhouse gas emissions report submitted to the UNFCCC is for the 1994 calendar year (10). In contrast, under the Montreal Protocol, China has reported annual detailed ozone-depleting substances consumption inventory data over 1990–2010. A rigorous system of surveillance—of policies, actions, and outcomes—could support a more robust international climate-policy regime. Conditioning international finance on participation in surveillance could increase the transparency of and trust in the global climate regime for all participants.

The Path Ahead

The outcome of the Durban negotiations has increased the likelihood that a sound foundation for meaningful long-term action can be developed. With the DPEA, there is a mandate for change. Governments around the world need fresh ideas, and they need those ideas over the next 2 to 3 years. Indeed, they have begun to solicit such ideas as the negotiators begin to frame the implementation of the DPEA.

How can an international agreement facilitate meaningful emission mitigation in developed and developing countries while meeting the UNFCCC’s principle of “common but differentiated responsibilities and respective capabilities?” How can an agreement best leverage public and private finance for investment in climate-friendly technologies and climate adaptation? How can market mechanisms broaden participation and deepen emission mitigation? How can an agreement improve transparency, improve trust, and thereby increase both participation and compliance among nations?

This is a time for innovative proposals for future international climate-policy architecture, not for incremental adjustments to the old pathway. We hope this call will be heard by researchers in universities, think tanks, and advocacy groups around the world.

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Supplementary Materials

www.sciencemag.org/cgi/content/full/337/6098/1043/DC1

10.1126/science.1223836

BIOCHEMISTRY

Spare the (Elastic) Rod

Philip C. Nelson

Physicists love emergence. From a welter of complex details about a system's constituents, simple and universal rules sometimes emerge that adequately describe the collective behavior of the components. Even if these rules are not completely universal, they often have only a few relevant parameters, a vast simplification compared to the many that describe the constituents individually. But as Vafabakhsh and Ha remind us on page 1097 of this issue (1), emergent behavior can conceal important aspects of a system. Using a beautiful application of fluorescence microscopy, the authors provide the clearest evidence to date that the elastic-rod model for DNA mechanics, an emergent description that works well on long length scales, breaks down on shorter length scales relevant to cell biology.

Emergence is often a function of increasing length scale. For example, the complex intermolecular dynamics of individual water molecules can be ignored in the design of plumbing; for this purpose, it suffices to know just two parameters for water: its mass density and its viscosity.

However, the very forgetfulness of nature that simplifies its long-scale character can also conceal the details that we need to know if we are to understand shorter-scale regimes.

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The mechanical properties of DNA are a case in point. It is tempting to regard this famous molecule as just a database containing the algorithm for constructing an organism. But DNA is also a physical object that constantly bends, twists, and interacts with other biomolecules. Particularly important, DNA is often observed to be tightly bent, in contexts such as gene regulation and packaging (see the figure).

Polymer physicists have long known that a stiff polymer like DNA will display emergence. On long length scales, such a molecule may be adequately described as an elastic rod, that is, a rod that resists bending with a linear relation (Hooke's Law). The mathematics of elastic rods was developed in the 19th century; all that is needed in the polymer context is to add the action of random thermal motion, which takes on crucial importance on the nanometer scale.

Some of the first single-molecule manipulation experiments on DNA found that the simple elastic rod model, despite having only a single free parameter, gave a quantitative account of the behavior of lambda phage DNA (2). This agreement became even more impressive with later experiments.

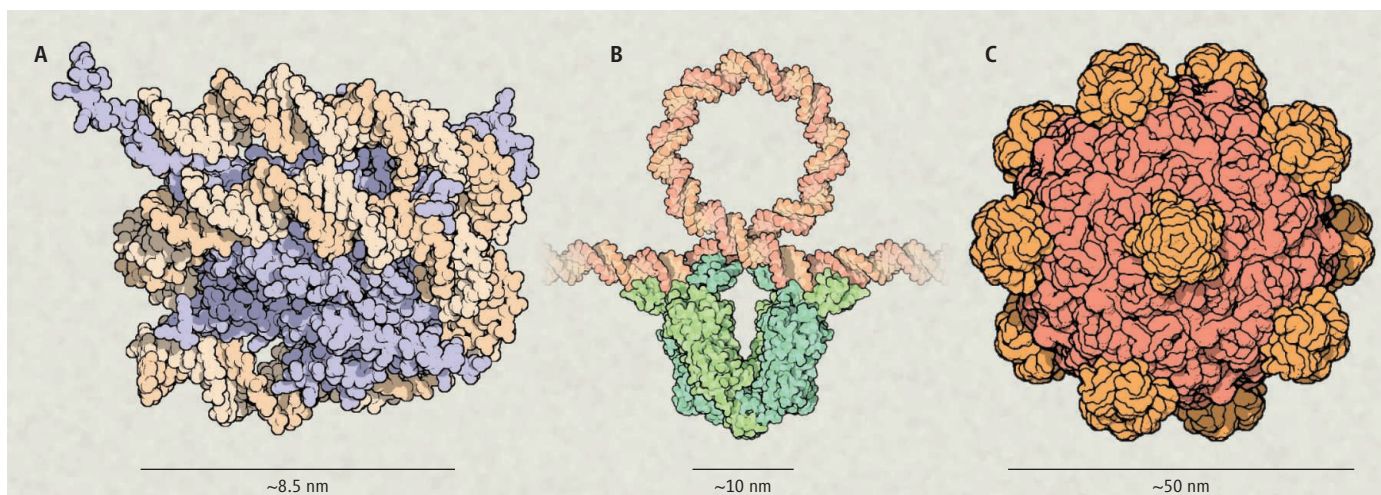
Could DNA be literally regarded as a linearly elastic rod? The late Jonathan Widom (3) did not think so. He knew that the rod model required a prohibitive amount of elastic energy to be expended to form the struc-

Experiments show that at biologically relevant length scales and under conditions resembling those in cells, DNA does not behave like an elastic rod.

tures shown in the figure; yet, these structures form readily.

To reduce uncertainties that arise from the complex cellular milieu, Cloutier and Widom undertook in vitro experiments with DNA fragments of length equal to the circumference of the nucleosome core particle. They assayed the ability of these fragments to form loops in the absence of the histone proteins that might be thought to facilitate loop formation. The results were astonishing. Not only did small loops form readily; for loops of biologically relevant sizes (see the figure), the ability to form spontaneously was found to be nearly independent of loop size (apart from a modulation with periodicity equal to the helical pitch) (4, 5).

Perhaps these results should not have come as a great surprise. It has long been known that DNA has discrete alternate conformations, attainable at a modest free-energy cost, including some with sharply localized kinks (6), locally melted regions, and flipped-out base pairs. Thus, just as bending a soda straw eventually gives a catastrophic breakdown of its rod elasticity, so too could severe nonlinearities enter DNA elasticity. Kinks were also known to form in tightly bent structures like the nucleosome (7, 8). Accordingly, immediately after Cloutier and Widom's work, theorists investigated simple models incorporating highly bendable behavior on short length scales (9, 10). Such models automatically



Biological examples of tightly bent DNA. (A) DNA winds around a protein core (lavender) to form the nucleosome. (B) A transcription factor (green) forces DNA into a tight loop. (C) A bacterial virus packs over 10,000 base pairs of DNA into a

small capsid. The results of Vafabakhsh and Ha bear on the question of how such structures can self-assemble despite the high elastic energy cost traditionally attributed to tightly bent DNA.

displayed emergent elastic-rod behavior on long length scales, thus reconciling the new experiments with the old ones. Later, a mesoscopic (intermediate-scale) model was found that incorporated still other experimental results, yet, like the original elastic rod, had only one free parameter (11).

Unfortunately, Cloutier and Widom's experiments were fraught with uncertainties. Their assay relied on the large ligase enzyme, required an intricate protocol, and did not directly report looping rates. Later experiments have given similar results without use of ligase (11, 12), but in each case, some aspect of the assay did not resemble the situation in vivo.

Vafabakhsh and Ha now offer a clean, simple demonstration of non-rodlike behavior in DNA at biologically relevant scales. The new work is done in vitro, and hence is free from many unknowns in the cell, yet was not

affected by some potential artifacts present in previous in vitro experiments, for example, the proximity of hard walls and large reporter beads. Not only do their results vindicate Widom's intuition; they also show that this behavior occurs for generic sequences [it is even more pronounced for special ones (13)]. Finally, the experiment confirms the near independence of looping ability on DNA length in the relevant regime—a cardinal property in the theories of (9–11).

The new results will still need to be integrated with previous experiments, not all of which have seemed to fit the picture described above (14). They will also provide guidance as theory seeks to go beyond generic models to ones predicting the details of sequence dependence. Already, however, they illustrate the two-edged character of emergence: It can simplify behavior, but this is not always appropriate. To learn about a system on some

length scale, we must devise experiments that specifically probe that particular scale.

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10.1126/science.1227014

CHEMISTRY

Vibrational Excitation Can Control Tropospheric Chemistry

Geoffrey Tyndall

Earth's atmosphere is often compared to a low-temperature combustion system. Reactive hydrocarbons are emitted from both natural and anthropogenic sources and then oxidized by hydroxyl radicals (OH) through a complex chain of reactive free radicals. It is usually assumed that the molecules and radicals involved are all in their lowest energy states. However, on page 1066 of this issue, Glowacki *et al.* (1) show that a strikingly different product distribution can be obtained in the oxidation of acetylene depending on whether the radicals contain high amounts of internal (vibrational) energy or are fully relaxed. The study used a combination of experiment and theory to provide a complete description of the reaction of OH radicals with acetylene in the presence of varying amounts of O₂ and N₂.

Acetylene (C₂H₂) is an important atmospheric constituent, being emitted as a by-product of incomplete combustion. It has an atmospheric lifetime of about 12 days (which is governed by its reaction with OH

radicals), which allows it to be used as a tracer of anthropogenic activity or biomass burning in relatively clean regions. Previous studies have shown that two sets of oxidation products are accessible under atmospheric conditions; one makes formic acid and formyl radicals, and the other makes glyoxal and reforms the hydroxyl radical (see the figure). The overall reaction rate constant was measured a number of years ago (2, 3) but during one of these studies, Bohn *et al.* (2) noted that the apparent rate constant for OH loss was reduced in the presence of oxygen. To explain the O₂ dependence of the rate constant, they suggested that the O₂ could interact with the energetic free radicals (R-OH) produced from the addition of OH to acetylene. Glowacki *et al.* convincingly confirmed this hypothesis by both experiments and theory.

Acetylene is a linear molecule with a triple bond between the carbon atoms. When OH adds to C₂H₂, the hydrogen atom on the opposite carbon can end up either on the same side as the OH or diametrically opposed. According to the calculations of Glowacki *et al.*, the configuration with the hydrogen furthest from the oxygen should

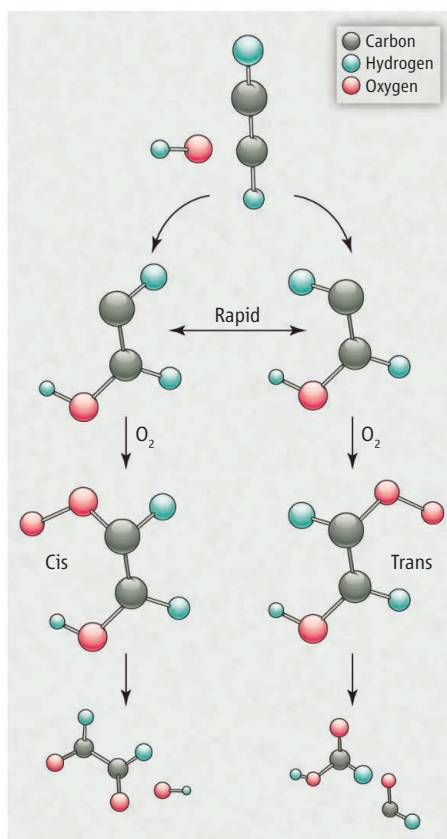
Which products form during the oxidation of acetylene by hydroxyl radicals and oxygen depends on the internal excitation of a radical intermediate.

be the most stable and leaves an unpaired electron next to the oxygen (the cis position). When O₂ adds to this unpaired electron, it can easily abstract the H atom on the OH group, providing a low-energy exit channel to glyoxal and OH. If the O₂ adds trans to the hydroxyl group, the rearrangement to products is a little more tortuous, but should also be rapid, leading to formic acid and HCO.

The two conformations of the radical do not interchange freely in the ground state but are separated by a small energy barrier. However, when the OH initially adds to the C₂H₂, the intermediate radicals have sufficient internal energy (in the form of vibrational excitation) such that the hydrogen can flip easily between the cis and trans configuration. However, collisions with other molecules transfer energy out of the radical, and after a sufficient number of collisions, the internal energy is reduced below the barrier. Thus, the more energetic the C₂H₂-OH radical is when it reacts with O₂, the more likely it is to produce formic acid, and vice versa.

The situation acquires added complexity when it is recognized that O₂ can act both as a collision partner and a reactive partner, whereas N₂ just acts as an inert collision

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Depends on how long the excitement lasts. The two paths of the oxidation of acetylene (C_2H_2) with hydroxyl radical ($OH\cdot$) and oxygen (O_2), an important atmospheric reaction studied by Glowacki *et al.*, are depicted. Initially the $C_2H_2\text{-}OH\cdot$ radical forms with a high degree of internal vibrational energy, and the cis and trans forms interconvert. If the reaction with O_2 occurs when this internal energy is present, roughly equal amounts of the two sets of products are formed. However, if collisions cool the intermediate sufficiently, the more stable cis form and the glyoxal pathway (that reforms $OH\cdot$) is favored.

partner. In their experiments, Glowacki *et al.* made high-quality measurements of OH radical decays in the presence of C_2H_2 over a wide range of temperatures (212 to 473 K) and pressures (13 to 100 mbar or 10 to 760 torr). They also varied the ratio of O_2/N_2 , confirming the observation of Bohn *et al.* (2) that the extent of OH regeneration depended on the relative amounts of these two gases, not the absolute amounts.

These observations strongly support the hypothesis that the nascent R-OH radicals were being intercepted by O_2 before they could be fully relaxed. The explanation was further supported by high-level quantum chemical calculations on the structures and energetics of the system, coupled with simulations of the transient energy flow in the radicals. These calculations followed the

evolution of the energy distribution among the $C_2H_2\text{-}OH$ radicals and their O_2 adducts, showing that at atmospheric pressure, the radicals typically took tens of nanoseconds to undergo vibrational relaxation. In accord with the experimental findings, at high O_2 content (90%), the radicals were intercepted before relaxation, leading to a roughly equal amount of each product, while at low O_2 (1%), the radicals were fully relaxed by collisions with N_2 , which led to a preponderance of glyoxal as product.

The work of Glowacki *et al.* shows that even at the relatively high pressures of the atmosphere, vibrational excitation may play a role in chemical reactions. Chemical activation is known to exert subtle effects on unimolecular processes, in the decomposition of organic alkoxy radicals (4), for example. However, it is unusual for a bimolecular reaction to be affected by the internal quantum state of a molecule. For example, the reactions of O_2 with these alkoxy radicals are normally assumed to proceed at the same rate, independent of the internal energy (but this assumption has never been verified).

The reaction of OH radicals with isoprene, which has the highest total emission rate into the atmosphere of any hydrocarbon except methane, also proceeds by an

addition mechanism analogous to the $OH\text{-}C_2H_2$ reaction. Because the OH-isoprene adduct can also exist in cis and trans forms, the products of isoprene oxidation might also be dependent on the interactions of the vibrationally excited R-OH radicals with O_2 (5). In fact, Dibble and co-workers recently completed a study of the isomerization rates of 1-methylallyl radicals, which can be considered prototypes for the isoprene OH system (6), paving the way for studies of more complex systems. Overall, it is likely that most of the reactions occurring in the atmosphere are dominated by thermal energy distributions. However, vibrational excitation like that seen by Glowacki *et al.* plays a role in some of the most important systems, and these systems will continue to provide exciting challenges to both experiment and theory in the coming years.

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10.1126/science.1227528

CELL BIOLOGY

Beyond Oil and Water—Phase Transitions in Cells

Anthony A. Hyman and Kai Simons

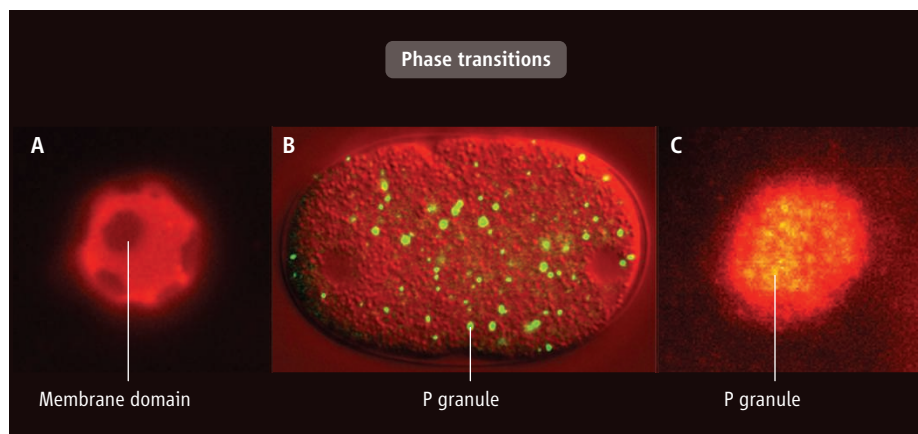
The organization of cellular compartments may be driven by liquid phase separations and the collective low-affinity interactions of macromolecules.

Contemporary biology has identified many proteins involved in different cellular processes, but we are far from understanding how they perform the tasks that cell functions require. How do collections of proteins and other molecules come together to form compartments (1) containing large numbers of macromolecular machines that execute specific and complex reactions? The search for underlying principles has been reinvigorated recently in

part by insights into the role of phase transitions in organizing cellular compartments.

The question of how biological macromolecules form organized assemblies was posed at the dawn of biochemistry in the early 20th century. A physico-chemical description of the cell was based on ideas from colloid chemistry to describe large-scale organization of macromolecules. Biologists considered the cytoplasm to be densely packed with liquid colloid particles that constituted a separate phase, distinct from the surrounding aqueous environment (2, 3). Such phase transitions were potentially powerful ways to segregate biological

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Phase transitions in the membrane and cytoplasm. (A) Membrane domains (dark regions) are shown in the plasma membrane of a rat basophilic leukemia cell. (B) P granules (labeled in green) in a *C. elegans* one-cell stage embryo are imaged by fluorescence microscopy, as described in (6). Liquid P granules consist of proteins and RNAs. (C) A single P granule in a *C. elegans* embryo is imaged by stimulated emission depletion microscopy, as described in (6). In both examples, the plasma membrane and P-granule compartments have been formed by a phase transition involving liquid-liquid demixing. Such demixing can be described by the Gibbs phase rule, which states that the number of demixed entities (P) for a system at equilibrium is strictly correlated with the number of chemically independent components (C) by the expression $P = C - F + 2$, where F is the number of independent variable properties (such as temperature or the fraction of phase components). Presumably, selective pressure has selected for certain P values that give small numbers of phases by ensuring that the different components are not chemically independent, and together form a collective in the condensed phase (13).

macromolecules. However, scientists of that era did not understand enough about macromolecules to connect them to the physical chemistry of the cell.

The molecular biology revolution radically changed the focus from a physicochemical description of the cell to the structure of individual macromolecules. Biologists concentrated on determining “lock-and-key” intermolecular interactions and the formation of protein complexes with defined molecular architecture. But detailed insights into the function of single macromolecules and macromolecular complexes have not been sufficient to understand cytoplasmic and membrane organization on larger scales. Currently, the ideas of colloidal chemistry and phase separations are reemerging to describe the organization of cellular biochemistry.

Phase transitions are common in the non-biological world. For instance, the transformation of water vapor to liquid drops after cooling, as seen on a car windshield on a cold morning, is a classic example of a gas-liquid phase transition. In biology, phase transitions can take the form of liquid-liquid demixing, where two liquids with different properties separate from each other. One example is the two-dimensional separation of lipids and proteins in membranes into dynamic liquid membrane rafts, distinct from the surrounding bilayer (4). The capability to form membrane domains is a subcompartmentalization device that allows for regulated pro-

tein segregation within the membrane plane. This mechanism is used to control endocytic or exocytic membrane transport, to transduce specific signals across the plasma membrane, or to carry out different biochemical reactions, depending on the proteins involved. Key to understanding the principles underlying liquid-liquid demixing in cell membranes is the mutual interactions between sterols, sphingolipids, and raft proteins (5). These form dynamic nanoscale assemblies that coalesce through multivalent interactions between raft lipids or between proteins into more stable platforms, which form a condensed, tightly packed, and ordered phase within the membrane.

However, liquid phase transitions are not confined to membranes. Cells have numerous examples of nonmembrane-bound compartments containing many proteins that perform complex biochemistry. These compartments form rapidly and are disassembled when not required. Examples are protein-RNA bodies such as Cajal bodies in the nucleus (implicated in RNA metabolism) or nuclear promyelocytic leukemia (PML) bodies that form under stress conditions in certain cells. Recent studies on P granules and nucleoli suggest that protein-RNA complexes are liquids that form by liquid phase transitions from cytoplasm. P granules are protein-RNA complexes that are involved in germline formation in the nematode *Caenorhabditis elegans*. These granules exhibit liquid-like behavior; that

is, they form fluid droplets (6), suggesting that they arise through liquid-liquid demixing from the cytoplasm (see the figure). Nucleoli, which are sites of ribosome synthesis, were also shown to behave like liquid droplets of protein-RNA complexes, exhibiting viscous-like fluid dynamics (7). A further discovery in the structure and function of ribonucleoproteins (RNPs) (8–10) demonstrated that in mouse brain and human cell extracts, proteins with low-complexity sequence domains (regions with low amino acid diversity) separate into a different phase together with RNA during liquid-liquid demixing. These studies suggest a model in which RNAs bind to RNA binding proteins, which in turn phase separate using their low-complexity sequences. The function of low-complexity sequence domains, which are abundant in the protein universe, have long puzzled biologists, but these experiments support the idea that they may have evolved to mediate such liquid-liquid demixing.

One problem in thinking about liquid phase separations in biological systems is the large number of components in a compartment. For instance, nucleoli are thought to have over 100 components. A possible framework for thinking about how different proteins contribute to the fluid state would be to consider three classes of molecules—for example, the multivalent, sometimes disordered proteins, the interaction of which forms the liquid phase in cells, or the ordered phase in membranes (10). Even in complex compartments, this type of molecule may be relatively few. In the case of P granules, although they are thought to contain dozens of components, two alone, when expressed in cultured mammalian cells, can form P granule-like structures (11). A second class of proteins make specific interactions with the first class. The third are molecules that partition selectively into the condensed phase formed by the first two classes of proteins and/or RNAs. In complex compartments, this third class is likely to be the most abundant and responsible for the localized biochemistry and its regulation.

In cells, the specific characteristics of liquid phase transitions will depend on the interactions between the molecules involved. Only recently has it been possible to reconstitute liquid-liquid demixing using *in vitro* systems (12) and multivalent signaling molecules. The proteins Nck and N-WASP, two molecules that interact with each other, when generated *in vitro* could form liquid droplets in which the concentration of the proteins in the drops was about

PHOTO CREDIT: M. GRZYBEK, K. SIMONS (PANEL A)

100 times that in the surrounding aqueous medium. Similar droplets were also observed when these proteins were overexpressed in cells. Notably, the concentration needed for the phase transition into fluid droplets correlated with the valency of these interacting proteins. The importance of this (12) and other *in vitro* work (8) is that it allows the study of the molecular basis of liquid phase separations in cells. It also shows that phase transitions in cytoplasm are not confined to assemblies that contain RNA, suggesting that phase separations could have a general role in organizing cellular biochemistry. There are many other candidate molecules with low-affinity polyvalency that could lead to liquid-liquid demixing, such as polyADP ribose, glycogen, and ubiquitin chains.

Taken together, these studies reveal a number of interesting features of liquid phase transitions in cells. They can occur in two or three dimensions, they involve the assembly of small macromolecular complexes through multivalent interactions, and they can form mesoscale to micrometer-scale fluid phases (13). Furthermore, high concentrations of solutes may also contribute to mesoscale organization in certain biological systems (14). The concentration of complexes that form the more condensed phase is apparently regulated close to the threshold of phase transition. This may reflect a general tendency of biological systems to be poised near a phase transition and thus promote large responses to small changes in

the environment (15). More generally, multivalent weak interactions between proteins, or proteins and RNAs, provide the properties for liquid-like states, perhaps explaining their prevalence.

The idea of liquid-like states that either separate from the cytosol or occur in cell membranes is a powerful way to think about cellular compartments (16). Changes in valency of interaction by regulatory events such as phosphorylation would allow a phase transition in which the components become rapidly concentrated in one place in the cell. Entry of proteins or other regulators into condensed phases could lead to fast disassembly of liquid compartments. A small increase in the concentration of components could allow reactions to start without any other regulatory event, as the concentrations rise above the Michaelis constant (K_m) for the reaction. Depletion of components from the cytoplasm as they segregate into the condensed phase could stop reactions in the cytoplasm. One could envisage developing drugs that partition directly into fluid phases, thus changing their separation behavior.

Many cellular compartments form rapidly and are disassembled when not required. Also, a surprising number of proteins involved in metabolism and stress responses form cytoplasmic puncta in yeast (17). It will be fascinating to examine each one of these compartments to ask whether their formation also represents examples of liquid phase separation, and then to deter-

mine the criteria for liquid-liquid demixing. More generally, phase transitions may have important implications in disease. Because they can undergo such large-scale changes in arrangement of molecules, defects in their organization are likely to have major effects on cell viability. For instance, the large number of protein aggregates seen in neurodegenerative disease could be a product of unwanted or misregulated phase transitions.

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1. Compartments can refer to organelles such as mitochondria, or nonmembrane-bound organelles such as nucleoli. More generally, compartments can also correspond to local concentration of molecules in specific biochemical processes such as P granules or stress granules and other subcompartments such as domains in cellular membranes.
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10.1126/science.1223728

ECOLOGY

Bad News for Soil Carbon Sequestration?

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Rising atmospheric carbon dioxide (CO_2) concentrations are expected to increase plant photosynthetic activity and the transfer of fixed carbon belowground, providing a potential buffering mechanism against elevated CO_2 (1). Arbuscular mycorrhizal fungi (AMF) are central to this potential extra carbon sequestration. AMF form symbioses with most land plants, in which the fungi

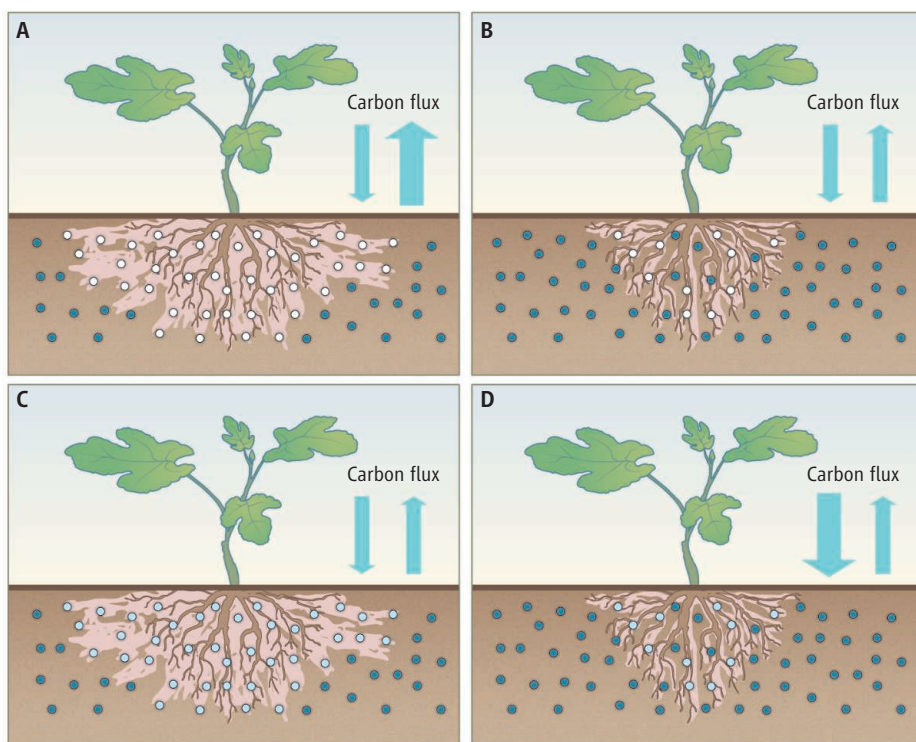
supply the plant with nutrients in exchange for carbohydrates (2, 3). But to what extent will this extra fixed carbon stay sequestered in the soil (1)? On page 1084 of this issue, Cheng *et al.* (4) show not only that the extra soil carbon is respired back to the atmosphere, but also that AMF activity stimulates additional decomposition of soil organic carbon. Increased carbon fixation by plants and transport of this carbon to the soil via AMF may thus result in a net source of CO_2 , rather than the sink we might have hoped for.

Plants may drive this AMF-dependent decomposition to gain access to nitro-

Arbuscular mycorrhizal fungi may stimulate additional decomposition of organic carbon in the soil, resulting in a net source of carbon dioxide.

gen from soil organic matter (4). Available nitrogen often limits gross primary production and growth responses to elevated CO_2 (5). Thus, increased translocation of nitrogen to the plant by AMF, specifically sequestered in the form of ammonium and not nitrate (4), may enhance plant growth. However, if enhanced plant growth leads to further increases in carbon transfer belowground, the net effect will be increased turnover of total carbon and nitrogen, rather than increased storage. Clearly, to understand the responses of terrestrial carbon cycling to climate change, interactions between the car-

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Carbon sink or source? Pink shaded areas are the zones in which soil saprotrophs are influenced by organic compounds exuded from roots or AMF. Dots represent patches of soil organic matter in which the organic matter remains intact (solid blue), partially degraded (lightly shaded), or fully decomposed (blank). Blue arrows depict the fluxes of carbon to and from the belowground compartment. The impact of soil microbes on carbon fluxes depends on the specific interactions involved in the plant-soil systems. (A) Efficient AMF colonization and efficient stimulation of soil organic matter decomposition, as observed by Cheng *et al.*, results in a net carbon source; (B) poor or no AMF colonization is carbon neutral; (C) poor stimulation of resident saprotrophs by AMF or presence of a poorly adapted saprotrophic community is also carbon neutral; and (D) no or poor AMF colonization and a poorly adapted saprotrophic community results in a carbon sink.

bon and nitrogen cycles must be taken into account (6).

Cheng *et al.* show that AMF stimulate increased decomposition of soil organic matter, especially under elevated atmospheric CO₂ conditions, but the mechanism behind this increase and the key players in these effects are not yet clear. Although AMF may play a direct role in carbon and nitrogen mineralization (7), these processes generally result from complex interactions between numerous bacterial and fungal species specialized in organic matter degradation (saprotrophs). AMF appear to increase decomposition of soil organic matter by stimulating the resident microbial communities, a classic case of the “priming effect” (8). In cases of such priming, soil microbes are stimulated by the addition of organic substrate, resulting in degradation of the added substrate and of additional organic matter in the soil. This leads to more soil respiration than can be explained by consumption of the added substrate.

Priming can be a very handy strategy for the degradation of organic pollutants,

whereby biostimulation of microbes by addition of substrate leads to increased pollutant degradation (9). However, it can have a negative effect on the retention of (supplemented) soil organic matter, which is essential for soil structure and quality (10), or in relation to mitigating rising atmospheric CO₂ levels.

The stimulation of decomposition by AMF observed by Cheng *et al.* presumably results from the activation of soil-borne microbes by the increased flux of AMF-derived substrates. This allows the newly stimulated soil microbes to degrade other organic matter sources in the soil (3, 11). AMF can influence soil bacterial communities, and elevated atmospheric CO₂ conditions have been shown to alter these interactions (3, 12, 13), but knowledge of the affected organisms and genes remains limited. These relationships will determine net carbon sequestration, making this knowledge essential for predicting the impacts of climate and land-use changes on the carbon balance of plant-soil systems.

AMF may impact soil carbon dynamics

in several ways. If soil microbes are well adapted to the stimulation by AMF-derived substrates and the degradation of organic matter species in the soil, AMF will induce a net loss of carbon from the soil, and this effect will be enhanced under elevated atmospheric CO₂ conditions (7) (see the figure, panel A). Without AMF colonization, or with maladaptive AMF associations (14), a more carbon-neutral situation may occur (panel B). Similarly, if soil saprotrophs are not well adapted to stimulation by AMF-derived substrates, or if they are ill-equipped to degrade the organic matter present in the soil, no net losses of carbon are expected (panel C). However, soil-borne saprotrophs are typically well equipped to degrade the types of organic matter most prevalent in their local habitats (15). The best scenario from the carbon sequestration perspective would be a combination of no or poor AMF associations with poorly adapted saprotrophs, leading to net carbon sequestration (panel D).

The findings by Cheng *et al.* may undermine some assumptions of plant-soil systems as potential sinks for atmospheric CO₂, but all is not lost. Through manipulations of soil nitrogen, organic matter quality, and field management, the balance of soil decomposition patterns can be tipped in a more positive direction. However, we require mechanistic understanding of decomposition processes and how they are influenced by changing environmental factors and land management. Interdisciplinary approaches that use the emerging environmental “-omics” toolbox, coupled with robust field and glasshouse experiments, seem to represent the best options for garnering this necessary knowledge.

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DEVELOPMENT

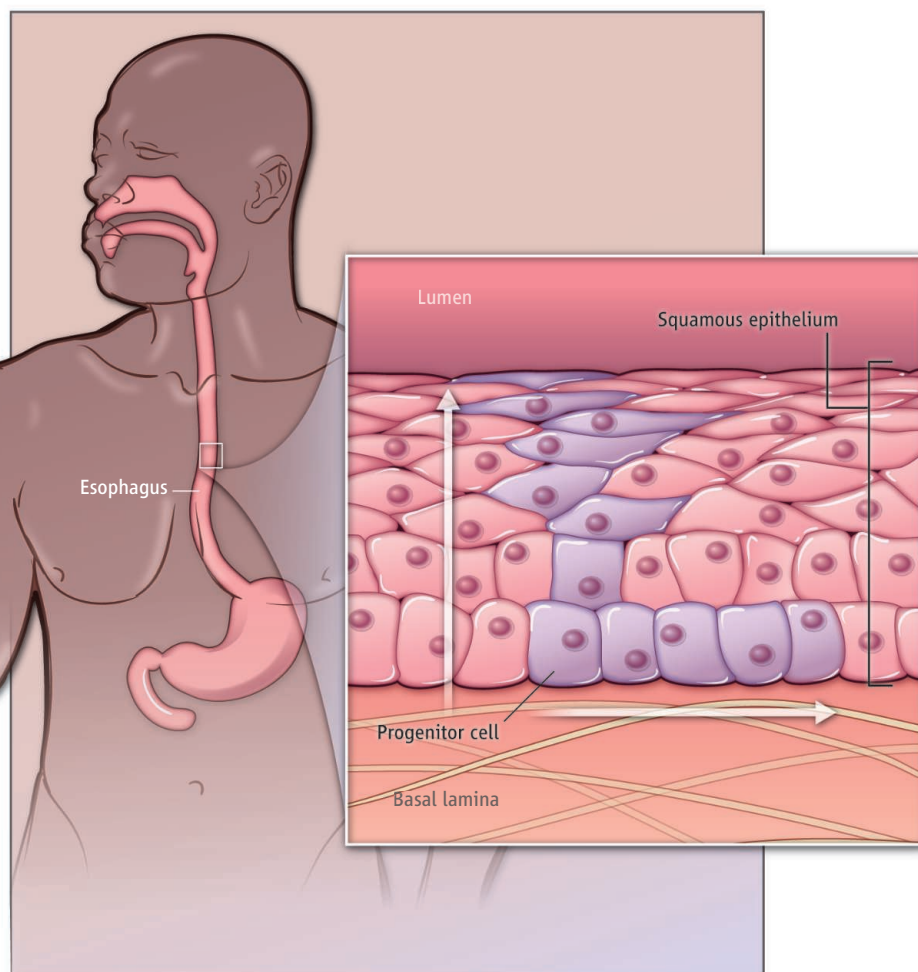
Esophageal Stem Cells, Where Art Thou?

Jake A. Kushner^{1,2}

The stem cell biology of the esophageal epithelium is an unresolved area in biomedical research. The field has major clinical implications, as esophageal cancer is a common cause of malignancy-related death, accounting for more than 500,000 deaths per year worldwide (1). Despite decades of research, there is little consensus regarding how this epithelium is maintained. On page 1091 of this issue, Doupé *et al.* (2) show that the esophageal epithelium is generated by a single population of cells that divide randomly into differentiated or proliferative progeny. Their finding contrasts with the prevailing hypothesis that stem cells underlie homeostasis of the tissue.

The esophageal epithelium constitutes the protective lining of the esophagus (see the figure). The tissue is composed of stratified squamous epithelial layers that arise from above the basal lamina (a basement membrane) and supra-basal cells (more superficial layers) (see the figure). In response to gastroesophageal reflux, the esophageal epithelium undergoes metaplastic change in which squamous epithelium is replaced by columnar and secretory epithelial cells. This condition, known as Barrett's esophagus, is associated with increased risk of esophageal cancer.

Surprisingly little is known about the lineage mechanism of tissue homeostasis in the epithelial lining of the esophagus. Studies of human tissue suggest that stem cells give rise to the esophageal epithelium through asymmetric cell divisions (3). Properties of putative progenitors from human esophagus have been characterized in vitro (4), and a population of cells isolated from the mouse esophagus could be expanded in vitro and contribute to esophageal epithelium when transplanted into injured tissue (5). But the



Replenishing the esophagus. The lumen of the esophagus is lined with a squamous stratified epithelium. In the model of Doupé *et al.* (based on the mouse esophageal epithelium), a single population of epithelial progenitor cells (purple) is present above the basal lamina. The epithelium is maintained, under basal conditions, by these cells, which divide and produce more progenitor cells, or differentiate into the layers of the stratified epithelium.

contribution of such candidate stem cells to normal or injured growth of the esophageal epithelium wasn't defined. As a result, their existence and functional importance has remained in doubt (6, 7). If stem cells don't maintain esophageal epithelium, what are the properties of the cells that do?

Doupé *et al.* determined the presence of populations of infrequently dividing cells in the mouse esophageal epithelium by using a technique that labels histones (proteins associated with chromatin in genomic

Are there stem cells in the esophagus or not?

DNA) in vivo. Unexpectedly, the only slow-cycling cells that retained the label were Langerhan's cells and T lymphocytes. These immune cells are likely present in the esophageal epithelium to perform immune surveillance functions, rather than tissue replenishment. Notably, candidate stem cell markers observed in previous studies in the esophagus or other tissues were not present in the label-retaining cells or other cells of the esophageal epithelium. This indicates that slowly dividing cells do not contrib-

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ute to the tissue homeostasis of esophageal epithelium, and further suggests that the tissue is somehow maintained by another lineage mechanism.

To further characterize the kinetics of maintenance, Doupé *et al.* labeled (using a genetic approach) a small number of cells in the esophageal epithelium and then followed their fate. Labeling in the basal lamina correlated with the total number of labeled suprabasal cells above, consistent with the idea that the esophageal epithelium is maintained by the division of cells above the basal lamina. The authors also waited for a period after inducing labeling before analysis and observed that the number of labeled cells in the basal lamina increased, implying that the original labeled cells divided to generate more such cells (and thus retained the label). These observations indicate that the epithelium is maintained by random cell division within an apparently homogeneous cell population above the basal lamina. This pattern is consistent with scaling behavior (i.e., scale invariance) observed in a power-law relationship, equivalent to the growth pattern of the interfollicular epidermis (8). Notably, these findings are inconsistent with a stem cell contribution to esophageal epithelium, in which case the clone size of

labeled cells would not have been expected to progressively increase.

Doupé *et al.* further investigated tissue homeostasis under dynamic (rapid cell growth) conditions. Although treatment of the mouse esophageal epithelium with retinoic acid (a growth-accelerating chemical) did cause an increase in the number of cells in the stratified epithelium, the lineage relationships of the epithelial cells were the same as those observed under normal conditions. These studies imply that a single population of esophageal progenitors maintains tissue homeostasis during normal and accelerated growth. Doupé *et al.* also modeled wound healing using an endoscopic biopsy technique. They observed a migrating front of proliferating cells that are clonally related, suggesting that highly proliferative cells within the epithelium can participate in tissue regeneration. Complementary histone-labeling studies suggest that virtually all nearby cells participate in regeneration. Wounding preferentially accelerated the cell division rate of these epithelial progenitor cells without altering differentiation. As a result, the authors make the surprising conclusion that wounding leads to an expansion of proliferating daughter cells, thus enabling a single population of epithelial

progenitors to effectively switch from normal tissue homeostasis to wound healing.

Homeostasis of tissues such as the esophageal epithelium remains an important field with major clinical implications. Given that basal and regenerative tissue homeostasis appears to be maintained by a single population of esophageal progenitors, a major challenge is to determine the safeguards that maintain normal growth in the esophageal epithelium, and to unravel how such mechanisms fail during oncogenic transformation in the development of esophageal cancer.

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Acknowledgments: I thank P. Kushner for helpful comments on the manuscript. J.A.K. is supported by the NIH (1R01DK064101, 1R01AG040110, P30DK079638), Johnson & Johnson, and the Robert and Janice McNair Foundation.

10.1126/science.1227506

CELL BIOLOGY

Mitochondrial Dynamics and Apoptosis—the ER Connection

Suzanne Hoppins and Jodi Nunnari

Mitochondria are endosymbiotic organelles that were pivotal in the evolution of eukaryotic multicellular organisms, enabling cells, through production of adenosine 5'-triphosphate, to overcome a steep energetic barrier (1). Another essential feature of multicellularity is programmed cell death or apoptosis—a process in which mitochondria also play a critical role. During intrinsic apoptosis, a signaling platform assembles on mitochondria that in some organisms is harnessed to permeabilize the outer mitochondrial membrane and release proapoptotic proteins. Assembly of this platform

is accompanied by dramatic changes in the dynamic behavior of mitochondria, which influence cell death. The dynamic properties of mitochondria are dependent on their division and fusion and govern the overall shape, connectedness, and distribution of mitochondria in cells. On page 1062 in this issue, Youle and van der Bliek (2) review the interplay between mitochondrial dynamics and mitochondrial quality-control and stress pathways. Here, we speculate on the role of mitochondrial division and fusion in the ultimate stress response, cell death. The recent discovery that the endoplasmic reticulum (ER), another ancient endomembrane organelle, actively participates in mitochondrial division has led to a new model linking mitochondrial dynamics and cell death. This suggests an unexpected convergence

Microdomains formed by the association of the ER with mitochondria during mitochondrial division may also be used to regulate cell death.

during evolution of mitochondria and ER—the two dominant endomembrane systems in eukaryotic cells that have previously been viewed as functionally distinct.

Mitochondrial division and fusion are mediated by the action of large self-assembling dynamin-related guanosine triphosphatases (DRPs) (3). Mitochondrial division is catalyzed by a single cytosolic DRP, DRP1, and fusion requires two integral membrane DRP families, MFN1/MFN2 and OPA1, which are distributed in the outer and inner mitochondrial membranes, respectively. DRP1 self-assembles into helical structures that wrap around mitochondria and coordinately divide the outer and inner membranes (4–6). Similarly, the self-assembly properties of mitochondrial fusion DRPs are also somehow harnessed for membrane

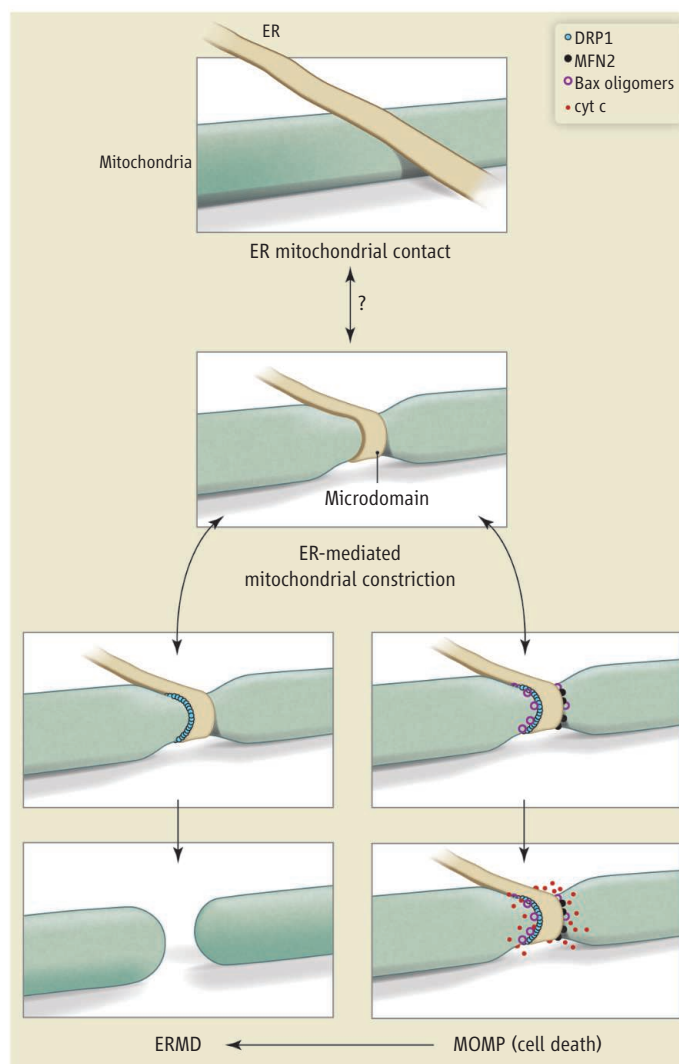
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tethering and lipid mixing of the outer and inner membranes (7, 8). In addition to their canonical roles in regulating mitochondrial structure, the mitochondrial division and fusion DRPs function in key quality-control and stress pathways. They impinge specifically on Bcl-2-dependent mitochondrial outer membrane permeabilization, which is required for apoptosis, suggesting that they directly link these two processes in the cell.

The exact mechanism by which mitochondrial DRPs and other mitochondrial shaping proteins influence outer membrane permeabilization is still a mystery. The best-characterized is the inhibitory role of OPA1, which is due in part to its regulation of the junctions positioned at the mouths of the internal mitochondrial compartments known as cristae, which act as gatekeepers in the release of proapoptotic proteins from the intermembrane space (9, 10). The positive regulatory role of DRP1 in mitochondrial outer membrane permeabilization depends on its recruitment to mitochondria. During apoptosis, DRP1 is massively recruited to the mitochondrial outer membrane where it assembles into foci, which mediate mitochondrial division, causing a dramatic fragmentation of the mitochondrial network. The proapoptotic Bcl-2 protein Bax behaves similarly to DRP1 during apoptosis; it is recruited to the mitochondrial outer membrane, where it inserts and oligomerizes to form foci that are functionally linked to outer membrane permeabilization. Under apoptotic conditions, DRP1 is found in foci with Bax on mitochondria (11). In healthy cells, MFN2 is also observed in foci on mitochondria and, similar to DRP1, under apoptotic conditions, is found in foci with Bax (11, 12). These apoptotic foci spatially mark mitochondrial constriction sites and mitochondrial tips, consistent with the idea that they are associated with the observed increase in mitochondrial division and fragmentation. However, extensive data show that mitochondrial fragmentation is not a key factor in mitochondrial outer membrane permeabiliza-

tion and cell death, indicating that the role of DRP1 in regulating outer membrane permeabilization is independent of its role in mitochondrial division per se. What, then, are the molecular roles of DRP1, MFN2, and other components that regulate mitochondrial dynamics, in apoptosis?

A clue to the functional importance of these striking cytological changes upon cell death, as well as the regulatory roles of mitochondrial dynamics components in mitochondrial outer membrane permeabilization, comes from the discovery that specialized ER tubules wrap around mitochondria and mark mitochondrial division sites (13).



ER-mitochondrial microdomains. A model showing how ER-associated mitochondrial division (ERMD) creates microdomains that can be harnessed for diverse cellular functions. The ER associates with mitochondria, marking sites of future mitochondrial division. These sites create microdomains enriched in mitochondrial division components, such as DRP1 and Mff. The microdomains may be used under stress conditions to recruit and regulate the activation of proapoptotic Bcl-2 proteins like Bax to promote permeabilization of the mitochondrial outer membrane (MOMP) and the release of death mediators, such as cytochrome c (cyt c).

One likely role of ER-associated mitochondrial division (ERMD) is to create a mitochondrial constriction site or geometric “hot spot” for the assembly of the mitochondrial division dynamin helix. It is possible that during apoptosis, ERMD also plays a critical role in Bax-dependent mitochondrial outer membrane permeabilization (see the figure). Specifically, ERMD sites could represent an ER-mitochondria microdomain that is critical for Bax insertion and oligomerization. The existence of such microdomains is substantiated by the observation that the DRP1 receptor and effector, Mff, accumulates at sites of ER-mitochondrial contact in the absence of

DRP1, providing a spatial mark for DRP1 recruitment to mitochondrial constriction sites (13). Microdomains generated at ER-mitochondrial contacts, called mitochondrial-associated membranes, have also been implicated in lipid and calcium exchange. The existence of specialized mitochondrial-associated membranes for Bax activation is supported by recent in vitro work demonstrating that sphingolipid metabolites derived from a non-mitochondrial membrane compartment directly stimulate the assembly and oligomerization of Bax in the mitochondrial outer membrane to promote permeabilization (14). In this context, an ER-mitochondrial microdomain would facilitate the shuttling of key lipid effectors of Bax.

Mitochondrial DRPs, and other mitochondrial and ER components, could both positively and negatively regulate mitochondrial outer membrane permeabilization by influencing the biogenesis and/or structure of the ERMD microdomain. The mitochondrial division and fusion DRPs are well suited to this role as they can assemble into an array of geometrically diverse membrane-associated scaffolds, which selectively recruit and spatially restrict lipid and protein effectors. Indeed, DRP1 may promote Bax oligomerization in vitro by stabilizing membrane tethering and promoting lipid mixing across membranes via hemifusion (15). In healthy cells, inactive soluble forms of Bax stimulate MFN2-dependent fusion (16). This find-

ing, coupled with the localization of MFN2 with Bax in foci during apoptosis, suggests an MFN2-dependent mitochondrial outer membrane permeabilization regulatory loop that impinges on ERMD domains.

The discovery of ERMD has revealed a pivotal regulatory hub in the cell. The mitochondrial dynamics components DRP1 and Mff, and MFN2, which helps mediate mitochondrial-ER contacts important for Ca^{2+} homeostasis (17), do not play essential roles in the biogenesis of ERMD domains (13). Thus, the structural components essential for their formation are unknown. However, the assembly, organization, and number of ERMD domains in a given cell are likely to be dynamic and thus could also dictate the progression of apoptosis. It is possible that

ERMD domains extend beyond the ER and mitochondrial outer membranes into the ER lumen and inner mitochondrial compartments, respectively, thereby integrating the functional status of both organelles. This is suggested by the regulation of mitochondrial outer membrane permeabilization by ER stress-induced apoptosis. Indeed, ER stress and mitochondrial dysfunction have been implicated in a shared set of diseases, such as neurodegeneration, which are associated with altered mitochondrial dynamics. This raises the intriguing possibility that alterations of ER-mitochondrial contacts may not only contribute to the normal regulation of cellular processes such as mitochondrial division and apoptosis, but may also be a contributory factor in disease (18).

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Acknowledgments: J.N. and S.H. are supported by the NIH (R01GM062942, R01GM097432, and K99HL103722).

10.1126/science.1224709

APPLIED PHYSICS

Structured Light Meets Structured Matter

Natalia M. Litchinitser

Metamaterials and singular optics are two fascinating branches of modern optics that until recently were rapidly developing in parallel yet independently. The former considers “simple” linearly or circularly polarized light or Gaussian beam propagation in “complex” materials with properties not found in nature. However, light can be a more complex phenomenon; in addition to conventional polarization states (spin), light beams can be radially or azimuthally polarized and carry orbital angular momentum (OAM). Structured light beams, containing phase or polarization singularities, enable properties and applications such as diffraction-free and self-healing propagation, single-molecule spectroscopy, nanoscale focusing, and even particle acceleration. A fascinating example of a beam carrying OAM is the optical vortex—a donut-shaped beam with a helical phase front (see the figure, panel A) (1–3).

The presence of a singularity can be observed by interfering a vortex beam with either a copropagating or tilted Gaussian beam, resulting in a spiral or fork-shaped interference pattern, respectively. Vortices

play an important role in a number of physical processes, ranging from microscopic structures of superfluid helium to macroscopic structures of tornadoes.

When light propagates in a vacuum or a homogeneous, isotropic, nondispersive transparent medium, both spin and orbital angular momentum are independently conserved. However, if the medium is more complex, either anisotropic or inhomogeneous, the spin or angular momentum can change, which leads to spin-orbit interaction. Such a spin-orbit interaction leads to the mutual influence of the polarization and the trajectory of the beam propagation, as revealed in the geometrical Berry phase (4), the topological spin transport or intrinsic spin Hall effect (5), and new regimes of nonlinear optical processes (6).

Metamaterials enable unprecedented control over light propagation, opening new avenues for using spin and quantum optical phenomena, and design flexibility facilitating new linear and nonlinear optical properties and functionalities, including negative index of refraction, magnetism at optical frequencies, giant optical activity, subwavelength imaging, cloaking, dispersion engineering, and unique phase-matching conditions for nonlinear optical interactions. Provided that metamaterials can be engi-

The synergy of complex materials and complex light is expected to add a new dimension to the science of light and its applications.

neered to realize nearly any imaginable optical properties, they are expected to change light-matter interactions of structural light. I will discuss examples of initial studies and outline directions where a synergy of the two fields may lead to a breakthrough.

The realization that the spin of photons provides an additional degree of freedom in nanoscale photonics led to the development of the field of spin optics (2). The possibility of using the plasmonic geometric phase was used to realize spin-dependent plasmonic focusing lenses (see the figure, panel B). Ultrathin metamaterial metasurfaces were also used to imprint abrupt discontinuities (vortices) on propagating light at mid-infrared frequencies (see the figure, panel A) (1). The recently demonstrated optical metasurfaces are likely to open new possibilities for the development of beam shaping and steering, plasmonic lenses, and other ultrathin components for optics on a chip (7).

In terms of signal processing, it was predicted that OAM could be used to encode information for quantum and classical systems (8). The combined use of different degrees of freedom of a single photon, such as spin and orbital angular momentum, enables the implementation of entirely new quantum information systems in a multidimensional space (9, 10). To date, most

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experimental studies, including field trials, were performed with wireless systems at radio frequencies. Can, then, OAM states be used for on-chip optoelectronic signal processing? This application requires the development of compact encoding and logic components, bringing us to the domain where metamaterials become indispensable.

So far, the discussion has focused on how metamaterials would modify light-matter interactions of structured light. However, singular optics can contribute to the devel-

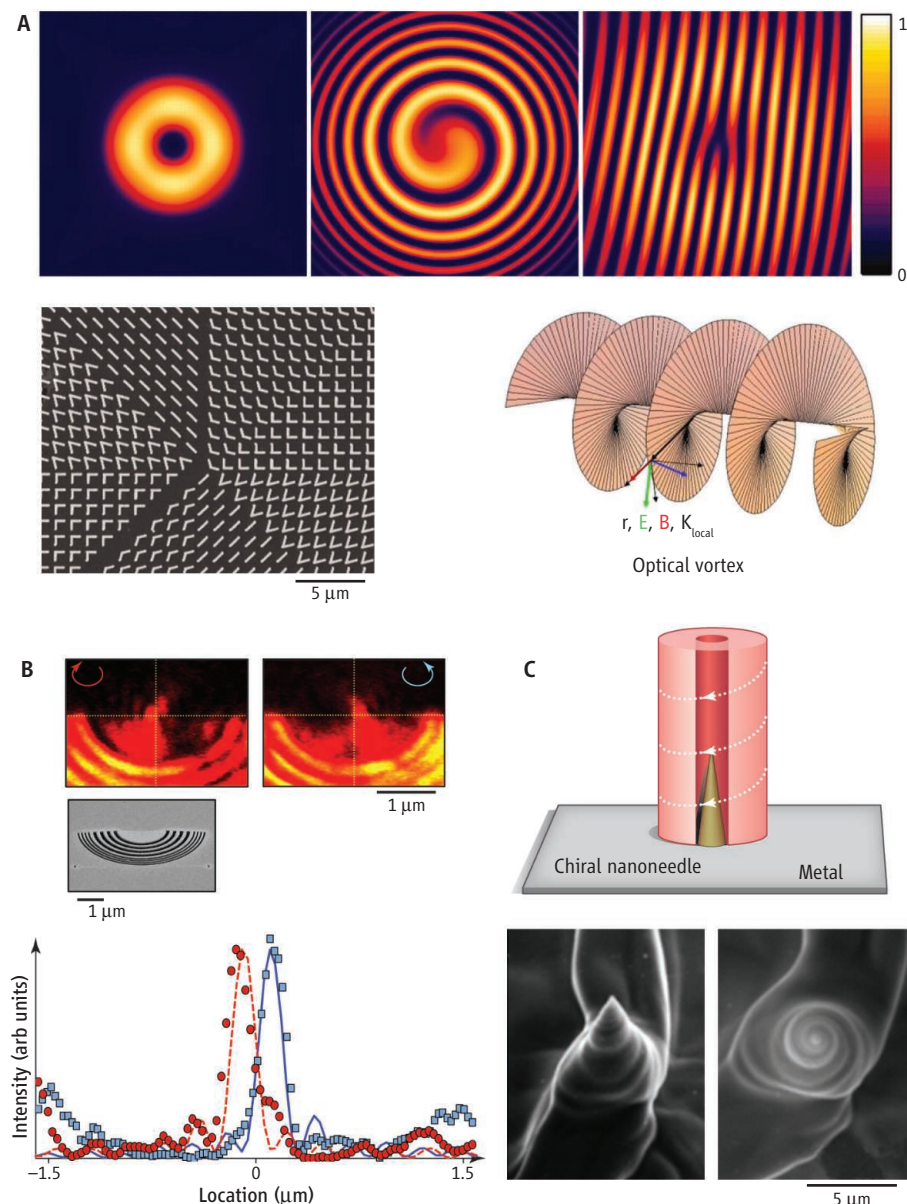
opment of complex metamaterial structures, as described in a recent demonstration of chiral metal nanoneedles formed by helicity transfer from vortex to metal (see the figure, panel C) (3). Chiral metamaterials enable nanoscale determination of the chirality and optical activity of molecules and chemical composites. Potential applications include nanoscale imaging, sensing, and optically active metamaterial surfaces.

Another area where reshaping of optical beams using metamaterials may play

a major role is light filamentation. Initial studies using vortex-preshaped femtosecond laser pulses indicate the possibility of achieving repeatable and predictable spatial and temporal distributions of the filaments (11). Metamaterials are expected to expand the capabilities of existing vortex optics for simultaneous control of intensity, polarization, dispersion, and phase properties of the beam for controlled formation of multiple filaments and filament arrays that would facilitate virtual waveguides for transporting and manipulating microwave radiation in air using filaments. Controlled filamentation of intense femtosecond pulses propagating in air may lead to various applications, such as remote sensing, light detection and ranging, and even lightning control.

Dispersion is one of the fundamental effects that describe the propagation of light in media, and it plays a key role in fiber-optic communication systems, nonlinear parametric interactions, supercontinuum generation, and solitons. The development of metamaterials with precisely tailored dispersion profiles will increase the efficiency of the spontaneous parametric down-conversion effect, an effect that was suggested as a source of high-dimensional states entangled in OAM (12).

Metamaterials are poised to bring new dimensions to the science and applications of complex light, including novel regimes of spin-orbit interaction, extraordinary possibilities for dispersion engineering, novel possibilities for nonlinear singular optics, trapping and optomechanical micromanipulation, as well as potential for applications in optical signal processing.



Light in a spin. (A) (Top) Calculated far-field intensity distribution of an optical vortex with topological charge one, spiral pattern created by the interference of the vortex beam and a copropagating Gaussian beam, and interference pattern with a dislocated fringe created by the interference of the vortex beam and a Gaussian beam tilted with respect to the vortex beam (1). (Bottom left) Scanning electron microscope image of a plasmonic metasurface that creates an optical vortex. (Bottom right) Helical wavefront [Courtesy of U. T. Schwarz]. (B) Spin-dependent plasmonic lens based on a geometric phase (2). (C) Optical vortex-controlled chirality of twisted metal nanostructures (3).

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10.1126/science.1226204

IBI* SERIES WINNER

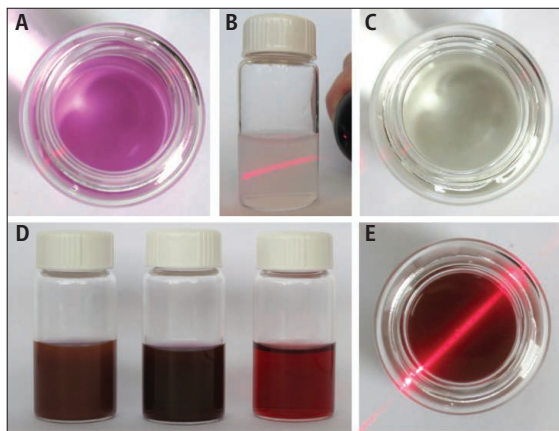
Discovering Nanoscience

A. Colin Blair, Ellen R. Fisher, Dawn Rickey*

The President's Council of Advisors on Science and Technology stresses the importance of adoption of empirically validated instructional practices, such as inquiry-based laboratory experiences, in higher education (1). The Exploring Gold Nanoparticles laboratory module employs an inquiry-based instructional tool called the Model-Observe-Reflect-Explain (MORE) Thinking Frame (2) to support student construction of evidence-based models of nanoparticles in introductory chemistry courses. Using MORE has been shown to enhance students' understanding of the nature of science and of scientific models compared with traditional teaching methods (3, 4).

The MORE Thinking Frame scaffolds students' thinking as they work to construct and evaluate evidence-based, molecular and/or nano-level models of chemical systems. A MORE module begins with a written, prelaboratory assignment (5) that prompts each student to describe his or her ideas about the system under study from macroscopic and molecular-level perspectives. This serves as the student's initial model. In writing their models, students are encouraged to reflect upon and articulate their own ideas, rather than to look up scientists' ideas. Next students conduct experiments in the laboratory (observe) and are explicitly prompted to reflect upon the implications of their observations as they relate to their initial model ideas. Students then refine their models and explain how their revised molecular-level ideas are consistent with the experimental evidence they collected.

After completing an iteration of MORE, students apply MORE to a subsequent set of laboratory activities, which provides addi-



Nanoparticles revealed. Using laser pointers, students explore various mixtures, collecting evidence to inform their models. A laser shines on (A) a KMnO_4 (aqueous) solution, (B) a fine suspension of AgCl in water, and (C) a HAuCl_4 (aqueous) solution. (D) The three gold nanoparticle mixtures that students synthesize. (E) A laser shines on one of the gold nanoparticle mixtures. When probed with the laser, scattering is not observed in the solutions (A) and (C), but is observed in the colloidal mixtures (B) and (E).

tional opportunities for them to refine their models. Each student presents a refined model, explains why it has (or has not) changed from his or her previous model, and proposes a generalized model that could be used to understand new situations. At the end of a module, each student proposes a next experiment that would help further refine or test his or her molecular-level model.

Although the MORE Thinking Frame is well-suited to guide students' thinking as they conduct original research, in our general chemistry laboratory course, we have more often applied it to investigations for which there is a fundamental, scientifically accepted model that has not yet been presented to students. Research has shown that instructional paradigms in which students first work to develop general rules or models, and expert ideas are presented only after students complete their investigations, promote deep understandings that facilitate transfer of learning [e.g., (6)]. Research in the context of another MORE module (7) indicates that student engagement in three thinking processes is strongly correlated with subsequent successful reasoning in new contexts. These include (i) constructing molecular-level models that are consistent with experimental evidence, (ii) reflecting accurately

Exploring Gold Nanoparticles, the IBI Prize-winning module, guides students' construction and evidence-based refinement of their personal models of gold nanoparticles.

and completely on how one's own molecular-level ideas changed relative to previous ideas, and (iii) identifying evidence to justify model refinements as part of the reflection on how and why ideas changed.

Exploring Gold Nanoparticles is a MORE module that guides students to construct and refine their own evidence-based models of the structure and properties of colloidal gold nanoparticle systems (5). The initial model assignment provides students with a chemical equation for the synthesis of gold nanoparticles and asks them to describe what they expect to observe and what they think will happen on the molecular level. At the beginning of the first laboratory session, a few students present their initial models to the class, and the instructor facilitates a discussion in which students share their ideas. The instructor does not contribute ideas, but encourages students to think about what evidence they can collect in the laboratory to test their models. The vast majority of students initially expect to observe the formation of a shiny, bulk gold precipitate when synthesizing gold nanoparticles.

During the module, small groups of students participate in several iterations of MORE. Students conduct experiments, reflect upon how the evidence collected relates to their models, participate in class discussions, and refine their models. In part I, students use laser pointers to observe the scattering of light in familiar aqueous solutions and colloidal mixtures (see photo, panels A and B) and interpret the evidence they collect to propose molecular-level pictures of each. Next, student groups synthesize gold nanoparticles, varying the amount of sodium citrate used such that different-sized nanoparticles are produced. Students again use laser pointers to collect evidence related to the nature of the reactants (C) and products (D and E in the photo), which are solutions and colloidal mixtures, respectively. An example illustrating how one student refined her model to be consistent with the evidence she collected is shown in section A of the table.

During part II, students view a live demonstration or computer animation of an atomic-force microscope (AFM). Groups are then provided with AFM images of the reaction mixtures from the previous experi-

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*IBI, Science Prize for Inquiry-Based Instruction; www.sciencemag.org/site/feature/data/prizes/inquiry/.

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ments, and students characterize the features of the images (e.g., the heights of the structures) and further refine their models of gold nanoparticles (sections B and C of the table).

In part III, students predict what will happen when aqueous solutions of potassium iodide and dextrose are added to a gold nanoparticle mixture (8). They then conduct experiments to test their predictions and further refine their models. Students predict what AFM images of their mixtures will look like and subsequently view and analyze those images.

In the final part of the module, students explore the use of gold nanoparticles as optical biosensors by designing a pregnancy test similar to a real-world urine test. The proposed mechanism behind the test is that higher levels of protein, such as the pregnancy hormone human chorionic gonadotropin, bind to the surface of gold nanoparticles and reduce salt-induced aggregation, which results in a color difference relative to lower levels of protein (9, 10). For this part of the module, each laboratory section is provided with 60 ml of a colloidal gold nanoparticle mixture and synthetic urine samples from two fictitious women (one pregnant and one not). The students must work together to design a pregnancy test that reliably distinguishes the samples.

Given students' initial lack of familiarity with nanoscience, there is great potential for them to develop an understanding of this topic in introductory science courses. The Exploring Gold Nanoparticles module

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A. Colin Blair earned his B.A. in Chemistry from Hendrix College, where he minored in English, and has a M.S. in Chemistry from Colorado State University. He has studied high-resolution infrared spectroscopy of transient species and students' beliefs about learning. **Ellen R. Fisher** is a Professor of Chemistry at Colorado State University where she studies materials chemistry, plasma science, and nanomaterials. She is interested in responsible conduct of research education and has developed inquiry-based materials for analytical chemistry courses. She is an associate editor of the American Chemical Society's ACS Applied Materials and Interfaces. **Dawn Rickey** is an Associate Professor of Chemistry at Colorado State University studying how people learn with a depth of understanding that enables them to apply scientific models effectively in new contexts. Rickey codeveloped the MORE Thinking Frame as part of her graduate work at the University of California, Berkeley.

effectively guides students to develop models of these systems. Most students successfully revise their initial models to be consistent with the evidence they collect, as well as with scientifically accepted views. In the process of constructing their own evidence-based models, as opposed to simply being presented with the expert model, students not only learn about the process of science,

but also enhance their understandings of the systems they study. This makes it more likely that students will be able to effectively build upon their models and apply them in new contexts, including more advanced courses and research.

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Acknowledgments: This work was supported by the NSF (DUE-0618829) and Colorado State University. We thank participating instructors and students; and C. J. Ackerson, S. Anthony, L. Dysleski, C. M. Elliott, F. Medhi, A. L. Prieto, B. A. Reisner, B. Reynolds, D. Roess, M. A. Teichert, L. T. Tien, and L. Wally for helpful discussions and use of equipment.

Supplementary Materials

www.sciencemag.org/cgi/content/full/337/6098/1056/DC1

10.1126/science.1215151

A. First Refined Model (following Part I: What are nanoparticles?)

"...After mixing, each of the solutions changed colors. [Mixture] A became a cloudy brown color (blue when held up to the light), [mixture] B became purple, and [mixture] C became red.... I had expected to see a gold precipitate, but nothing was visible. However, we were able to see the laser beam through all of the solutions, which tells me there had to be particles floating in the solution for the laser to reflect off of (even though we can't see them).... I thought all of the solutions would be the same and would produce a gold precipitate of the same amount. We did not find the solutions to be the same. Due to the three different colors, there appears to be 3 different substances formed. As for the gold, I believe that it doesn't just separate into individual gold molecules. I think the Au molecules cluster together while floating and that these clusters are the 'nanoparticles.' This explains why we can see the laser beam in the solutions—the clusters of Au molecules give the beam something to bounce off of so we can see it.... I think the laser is bouncing off clusters of particles that are bigger than regular molecules."

B. During-Lab Reflection (following Part II: Using AFM Images to Refine Your Model)

"The citrate was the only thing that was varied so that means the citrate effects [sic] the way the gold clumps together. In [mixture] A, they were the biggest clumps, in C they were the smallest.... The particles are bigger than a single gold atom, which means it is a clump of many gold atoms."

C. Final Refined Model (following completion of all parts of the module)

"...I thought all of the solutions would look the same with the AFM. However, this was wrong.... I believe the nanoparticles are actually clumps of Au molecules and the bigger ones are just more Au molecules clumped together...."

Student model excerpts. Excerpts from one general chemistry student's laboratory reports showing how she refined her model of gold nanoparticles.



EDUCATION

Can a New Vision Bring New Life to Biology Class?

As the fall semester begins at U.S. high schools and universities, the rites of introductory biology begin anew: Tens of thousands of students are listening to lectures on photosynthesis, memorizing parts of the cell, and learning the terms of taxonomy. The lessons would be familiar to their parents, in some cases even to their grandparents.

And that, experts say, is the problem. While biological research is advancing at warp speed, amassing new insights and new data as the lines separating biology, chemistry, mathematics, and engineering dissolve and the fields converge, biology education has seemed stuck in the 20th century. Now, urged on by science and education leaders—and many teachers—a growing number of schools are taking a new approach.

In the place of courses based solely on lectures and memorization, they are incorporating the latest practices of biological research, engaging students with the opportunity to think and work like scientists on issues with real-world relevance.

“We need education that will excite and inspire young people who will go on to become scientists and workers in biology-related fields,” said Alan I. Leshner, the chief executive officer of AAAS. “At the same time, we need to give all students a coherent view of the processes of life so that they’ll understand issues in their own lives and communities—issues like health, environmental protection, and biosecurity.”

Leshner and others say that a transformation in biology education, from elementary school through graduate school, will be

essential to support biotechnology, biomedicine, and other sectors that will be centers of 21st-century innovation and economic growth. Without that, the risk is that U.S. leadership in these fields will diminish, at great economic cost.

In the past 25 years, some two dozen major reports have focused in part or in full on improving biology and related science education. But in recent years, the idea has moved closer to critical mass.

Nobel laureate Phillip A. Sharp of MIT co-chaired the National Research Council committee that produced *A New Biology for the 21st Century*, published in 2009. Now the president-elect of AAAS, Sharp predicts that biology will be crucial in addressing global challenges in climate change, food security, energy, and health.

In an interview, Sharp said that to build new understanding of tumors or ecosystems, scientists must be able to analyze oceans of new data generated by genomic sequencing, imaging, and other advanced technology. Upper-level science and premed students will need skills in writing computer programs, working with databases, and analyzing statistics.

Even in Biology 101, “you have to introduce material that illustrates the importance of this emerging power in biological science,” Sharp said. “Ideally, with the right support system or online system ... you can get students to do simple computational problems.”

A broad effort under way since 2006 embraces the idea that undergrad students should be exposed to the real practice of

science. The project, overseen by Yolanda George, deputy director of AAAS Education and Human Resources, in collaboration with the National Science Foundation (NSF), the National Institutes of Health (NIH), and the Howard Hughes Medical Institute (HHMI), brought hundreds of educators, students, policy-makers, scientists, and others together for a series of regional and national meetings on transforming biology education.

The resulting 2011 report—*Vision and Change in Undergraduate Biology Education: A Call to Action*—offers a detailed, evidence-based agenda for transforming the curriculum and the culture. Among the key recommendations: Guide students to understanding of core concepts in courses that “are active ... inquiry-driven, and relevant,” featuring research experience as “an integral component.”

Too often, faculty use teaching methods that do not match the cutting-edge character of their biological research, said Terry Woodin, a program director in NSF’s Division of Undergraduate Education. In the Vision and Change meetings, she said, the message from students was clear: If you want to inform and inspire, lectures and memorization alone can be counterproductive.

“Most students are savvy users of the Web, so there’s not so much need anymore to memorize everything,” Woodin explained. “They want to feel that they’re part of the science community, and that they’re learning things that can be related to the real world. They want to be challenged to *think*.”

CREDIT: COLLAGE FROM SCOTSPENCER/ISTOCKPHOTO.COM (LEFT) AND WAIKIKI/ISTOCKPHOTO.COM (RIGHT)

Instructors, obviously, are crucial to the transformation of biology education. Through the BEN Scholars (BEN is short for BiosciEdNet), AAAS and its partners in the program are training select faculty members in the use of resources from the BEN Portal digital library; they bring their new insight not only to students but also to other faculty at their institutions. The Partnership for Undergraduate Life Sciences Education—PULSE—is a new effort by NSF, NIH, and HHMI that plans to enlist 40 Vision and Change Leadership Fellows to implement findings from the 2011 report.

And Project 2061, the AAAS science literacy initiative, is working with teachers in Colorado, Maryland, Boston, and Washing-

ton, D.C., on a module that prepares middle-school students for high school biology—by teaching them chemistry.

Director Jo Ellen Roseman said the lessons focus on polymer formation, a central process in sustaining life. Students use time-lapse photos to explore growth in animals. They see how mixing two colorless chemical solutions can yield nylon fibers; then they use Lego blocks and other models to visualize biological growth at the molecular level.

“My mission was to see if we could get kids understanding that biology is chemistry,” Roseman explained. Among a small group of teachers involved in the project last spring, “all got significant learning gains,” she added. “It’s very exciting.”

Of course, there will be obstacles to the transformation of biology education. Shirley Malcom, director of AAAS Education and Human Resources, says many of them are cultural—old habits die hard. But as more educators embrace the new ideas, she says, the Vision and Change project is entering a new phase: It will assess how the report’s recommendations are being adopted and which of them are proving effective.

“Change is hard and transformations don’t happen overnight,” Malcom said. “It’s a process—we need to try new things, refine them, and keep working at them. The hope is that all of these efforts will help people to see the great potential of these new ideas.”

U.S. ELECTIONS

Scientists Urge Obama, Romney to Address Key S&T Issues

AAAS has joined more than a dozen leading U.S. science and engineering organizations in preparing a list of science questions that they say President Barack Obama and Republican challenger Mitt Romney should debate in the 2012 campaign.

Culled from thousands of suggestions gathered by the nonprofit advocacy organization ScienceDebate.org, the final 14 questions posted at the organization’s Web site cover topics including innovation and international competitiveness, climate change, energy policy, ocean health, and the future of space exploration. The full set of questions has also been sent to the Obama and Romney campaigns.

While many of the questions invite the candidates to give a broad overview of their policy positions, others are quite specific. Regarding high school science test scores, one question asks: “In your view, why have American students fallen behind in the last three decades?” A climate change question asks: “What is your position on cap-and-trade, carbon taxes, and other policies proposed to address global climate change?”

The presidential candidates in 2008 answered a similar set of questions compiled by ScienceDebate.org, although they declined to participate in a full debate advocated by the organization. Shawn Lawrence Otto, the CEO and co-founder of ScienceDebate.org, said at least one of the 2012 presidential debate moderators has agreed to consider the 14 questions as they assemble their topics.



Otto feels that a format change, dedicating blocks of 10 to 15 min to specific issues in two of the debates, makes it more likely that the candidates will face one of the science questions.

“The old model was that science was this cloistered activity set apart from the national dialogue,” he said. But “our polling shows that 85% of voters across party lines think these issues should be debated by the candidates.”

Separately, the AAAS Office of Government Relations has developed a Web site that describes and tracks the candidates’ positions on science, technology, and innovation issues. The site—<http://elections.aaas.org>—focuses on the candidates’ policies on competitiveness and innovation; science, technology, engineering, and mathematics education and the workforce; climate and energy; health and medical research; and national security.

—Earl Lane and Becky Ham

ELECTIONS

AAAS Annual Election: Preliminary Announcement

The 2012 AAAS election of general and section officers is scheduled to be held in November. All members will receive a ballot for election of the president-elect, members of the Board of Directors, and members of the Committee on Nominations. Members registered in more than one section will receive election ballots for each section they are enrolled in.

Candidates for 23 of the 24 section offices are listed on the following pages. Additional names may be placed in nomination for any office by petition submitted to the Chief Executive Officer no later than 28 September. Petitions nominating candidates for president-elect, members of the Board, or members of the Committee on Nominations must bear the signatures of at least 100 members of the Association. Petitions nominating candidates for any section office must bear the signatures of at least 50 members of the section. A petition to place an additional name in nomination for any office must be accompanied by the nominee’s curriculum vitae and statement of acceptance of nomination. Biographical information for the following candidates will be enclosed with the ballots mailed to members in November.

Slate of Candidates

SECTION ELECTIONS

Agriculture, Food, and Renewable Resources

Chair Elect: Sally Mackenzie, Univ. of Nebraska-Lincoln; Michael F. Thomashow, Michigan State Univ.

Member-at-Large of the Section Committee: Richard A. Dixon, Samuel Roberts Noble Foundation; Pamela C. Ronald, Univ. of California, Davis

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Council Delegate: Clark Spencer Larsen, Ohio State Univ.; Dolores R. Piperno, Smithsonian National Museum of Natural History

Astronomy

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Council Delegate: Eugene H. Levy, Rice Univ.; Douglas O. Richstone, Univ. of Michigan

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Mitochondrial Fission, Fusion, and Stress

Richard J. Youle^{1*} and Alexander M. van der Bliek^{2*}

Mitochondrial fission and fusion play critical roles in maintaining functional mitochondria when cells experience metabolic or environmental stresses. Fusion helps mitigate stress by mixing the contents of partially damaged mitochondria as a form of complementation. Fission is needed to create new mitochondria, but it also contributes to quality control by enabling the removal of damaged mitochondria and can facilitate apoptosis during high levels of cellular stress. Disruptions in these processes affect normal development, and they have been implicated in neurodegenerative diseases, such as Parkinson's.

Mitochondria are double-membrane-bound subcellular organelles that provide a host of metabolic functions, including energy production through oxidative phosphorylation. Mitochondrial morphologies vary widely among different cell types. Fibroblast mitochondria, for example, are usually long filaments (1 to 10 μm in length with a fairly constant diameter of ~ 700 nm), whereas hepatocyte mitochondria are more uniformly spheres or ovoids. When mitochondria are viewed in live cells, it becomes immediately apparent that their morphologies are far from static. Their shapes change continually through the combined actions of fission, fusion, and motility. Rapid fission and fusion of mitochondria in cultured fibroblasts allows for the complete redistribution of mitochondrial green fluorescent protein (GFP) from one mitochondrion to all the other mitochondria of a cell within an hour. The wide range of mitochondrial lengths observed in different cell types and under different conditions results from changes in the balance between the rates of mitochondrial fission and fusion. Here, we discuss how fission and fusion contribute to mitochondrial quality control and the responses of mammalian cells to stress.

Mitochondrial Fusion and Fission Proteins

Mitochondrial fission and fusion processes are both mediated by large guanosine triphosphatases (GTPases) in the dynamin family that are well conserved between yeast, flies, and mammals (1). Their combined actions divide and fuse the two lipid bilayers that surround mitochondria. The mitochondrial inner membrane, which encloses the matrix, is folded into cristae that contain membrane-bound oxidative phosphorylation enzyme complexes and the bulk of the soluble electron transport proteins such as cytochrome c, whereas the smooth mitochondrial outer mem-

brane encapsulates the inner membrane and an intermembrane space.

Fission is mediated by a cytosolic dynamin family member (Drp1 in worms, flies, and mammals and Dnm1 in yeast). Drp1 is recruited from the cytosol to form spirals around mitochondria that constrict to sever both inner and outer membranes. Yeast share with mammals this core function of Drp1 but have distinct accessory proteins. Mdv1 recruits Dnm1 to mitochondrial fission sites in yeast, whereas Mid49, Mid51, and Mff recruit Drp1 to mitochondria in mammals (2), often at sites where mitochondria make contact with the endoplasmic reticulum (3). Fusion between mitochondrial outer membranes is mediated by membrane-anchored dynamin family members named Mfn1 and Mfn2 in mammals, whereas fusion between mitochondrial inner membranes is mediated by a single dynamin family member called Opa1 in mammals. Mitochondrial fission and fusion machineries are regulated by proteolysis and posttranslational modifications (1).

Mitochondrial fission is essential for growing and dividing cells to populate them with adequate numbers of mitochondria. It has been less clear why mitochondrial fission and fusion are also needed for nonproliferating cells, but the importance of these processes is evident from nonproliferating neurons, which cannot survive

without mitochondrial fission, and from two human diseases, dominant optic atrophy and Charcot Marie Tooth disease type 2A, which are caused by fusion defects. The importance of mitochondrial fusion for embryogenesis was shown with Mfn1 and Mfn2 knock-out mice, which die in utero at midgestation because of a placental deficiency, whereas the Mfn1 Mfn2 double knock-out mice die even earlier in development (4). Mouse embryo fibroblasts (MEFs) derived from the double knock-out mice do survive in culture, despite a complete absence of fusion, but some of their mitochondria display a reduced mitochondrial DNA (mtDNA) copy number and lose membrane potential, causing problems with adenosine triphosphate (ATP) synthesis (5). Mitochondrial fusion is therefore not absolutely essential for cell survival in vitro, but it is required for embryonic development and for cell survival at later stages in development (4). These differential requirements for fusion may stem from higher demands on oxidative metabolism in different cell types or on other functions that are indirectly affected by fusion, such as mitochondrial motility in neurons.

Fusion Promotes Complementation Between Damaged Mitochondria

Mitochondria have their own small circular genomes, encoding select subunits of ATP synthesis and electron transport proteins that form oxidative phosphorylation complexes with other subunits encoded by the nuclear genome, as well as transfer and ribosomal RNAs (tRNAs and rRNAs) needed for their translation. A single somatic cell can have thousands of copies of these genomes, which are grouped in protein-rich complexes called nucleoids, with between one and eight genome copies per nucleoid (6). Mutations and deletions that occasionally arise in mitochondrial DNA yield a heteroplasmic mixture of wild-type and mutant mitochondrial genomes within one cell. Maternal inheritance of these mutations can cause mitochondrial diseases, such as mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS) and myoclonus

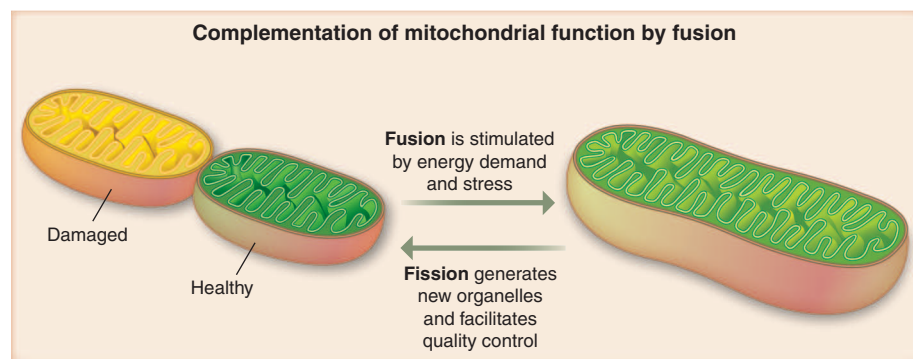


Fig. 1. Fusion rescues stress by allowing functional mitochondria (green) to complement dysfunctional mitochondria (yellow) by diffusion and sharing of components between organelles. Stress-induced hyperfusion yields maximal potential (light green), whereas under relaxed conditions cells are able to segregate the damaged (yellow) ones.

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epilepsy with ragged-red fibers (MERRF). Fortunately, mitochondria with mutant DNA can still fuse with other mitochondria in the same cell, allowing mitochondria with wild-type DNA to compensate for defects in mitochondria with mutant DNA by sharing components as long as the mutation load remains below 80 to 90% per cell (7, 8). Because nucleoids do not appear to exchange DNA (6), mitochondria in heteroplasmic cells complement one another by sharing RNA or protein components. Fusion between mitochondria can also rescue two mitochondria with mutations in different genes by cross-complementation to one another, and it can mitigate the effects of environmental damage through the exchange of proteins and lipids with other mitochondria. Mitochondrial fusion can therefore maximize oxidative capacity in response to toxic stress, as long as the stress is below a critical threshold (Fig. 1).

Mitochondrial Morphology Is Controlled by Metabolism

Rates of mitochondrial fission and fusion respond to changes in metabolism. Mitochondria become more fused when they are forced to rely on oxidative phosphorylation by withdrawing glucose as a carbon source (9). Increased fusion may be necessary to maximize the fidelity for oxidative phosphorylation by stimulating complementation among mitochondria (Fig. 1). Fusion is also enhanced by treatments that directly or indirectly inhibit protein synthesis and by starvation and mTOR (mammalian target of rapamycin) inhibition-induced autophagy (10–12). Starvation-induced autophagy may enhance fusion by increasing the reliance on oxidative phosphorylation through the metabolism of lipids and proteins (9). Alternatively, starvation may evoke a specific stress response called stress-induced mitochondrial hyperfusion (10), or it may inhibit fission to protect mitochondria from autophagic catabolism when they are most needed (11, 12). Each of these effects is consistent with a model in which mitochondrial dynamics help maximize the capacity for oxidative phosphorylation under stressful conditions (Fig. 1).

Repairing Small Amounts of Mitochondrial Damage

Mitochondria continually produce highly reactive superoxide anions as a byproduct of electron transport during oxidative phosphorylation. These reactive oxygen species (ROS) damage proteins, lipids, and DNA (Box 1). Damage to proteins in the electron transport chain may worsen the situation by producing even more ROS (13). Mitochondria use quality-control proteases to eliminate damaged proteins (14)

and respond to unfolded protein stress in the matrix through transcriptional induction of chaperone expression (15). Damaged mitochondrial outer membrane proteins also may be removed by the ubiquitin proteasome quality-control pathway (16). Mitochondria respond to genotoxic damage by some, but not all, of the DNA repair pathways found in the nucleus. These proteotoxic and genotoxic damage-response pathways target individual molecules for quality control, thereby rescuing mitochondria with minor damage without the need for altered fission or fusion rates (14). Another level of quality control entails the wholesale elimination of mitochondria by autophagy, a process that is linked to mitochondrial fission and fusion.

Scrapping Mitochondria That Are Beyond Repair

Autophagy is a well-established mechanism to compensate for nutrient depletion by degrading cellular components and to protect cells from del-

test” that could push a daughter mitochondrion to completely depolarize if it functions suboptimally. Mitophagy could be prevented with a dominant-negative mutant of Drp1, suggesting that fission is required for mitophagy (19). Photodamaged mitochondria undergo selective mitophagy (20), which is also consistent with the model that fission provides a form of quality control by segregating damaged parts of mitochondria and targeting them for elimination by autophagy (Fig. 2).

Recent work on two gene products mutated in familial Parkinson’s disease, PINK1 and Parkin, yields insight into a molecular mechanism of quality control via the elimination of damaged mitochondria (Fig. 3). The abundance of the kinase PINK1 is constitutively repressed in healthy mitochondria by import into the inner mitochondrial membrane and degradation by the rhomboid protease PARL. When a mitochondrion becomes uncoupled, protein import to the inner mitochondrial membrane is prevented so PINK1 is diverted from PARL and accumulates on the outer mitochondrial membrane. This yields a sensor of mitochondrial damage that can flag an individual impaired mitochondrion in a milieu of healthy ones. PINK1 on a damaged mitochondrion, through its kinase activity, recruits the E3 ligase Parkin from the cytosol specifically to that impaired mitochondrion (Fig. 3). Once there, Parkin ubiquitinates outer mitochondrial membrane proteins and induces autophagic elimination of the flagged mitochondrion (21).

This molecular pathway fits nicely with the fission model (19) (Fig. 2) to yield the mitochondrial quality-control model (Fig. 3). However, mitochondria have to be severely depolarized to accumulate PINK1, and the degree to which this happens physiologically is not clear. At least in cultured tumor cells that can maintain robust

ATP levels by glycolysis, mitochondrial F_1F_0 ATPase can cleave ATP derived from glycolysis and reconstitute membrane potential despite the complete loss of membrane potential maintenance through respiration (22). Furthermore, mitochondrial fusion as discussed previously can lead to compensation for missing components, thereby rescuing impaired organelles. These forces would be expected to counteract damage-induced depolarization of mitochondria and mitigate PINK1-mediated mitophagy. The stress test on membrane potential during fission (Fig. 2), however, might overcome those forces to trigger complete depolarization.

Mutations in PINK1 (23) and Parkin (24) lead to early-onset autosomal recessive Parkinson’s disease, suggesting that defects in mitochondrial quality control could cause certain forms of parkinsonism and supporting more general models that mitochondrial dysfunction is an etiology of

Box 1. Mitochondrial Stress

Various insults can cause damage:

- Environmental (radiation, toxic chemicals)
- Genetic (mutations in genes for metabolic processes or repair pathways)
- Spontaneous (ROS generated as byproduct of electron transport)

Types of damage:

- DNA
- Proteins
- Lipids

Problems caused by damage:

- Loss of metabolic functions (ATP synthesis, etc.)
- More ROS made by defective mitochondria
- F_1F_0 -ATPase may, instead of making ATP, consume ATP to generate membrane potential

Cellular responses to damage:

- DNA repair
- Proteases
- Lipases
- Mitochondrial unfolded protein response
- Mitophagy
- Apoptosis

eterious protein aggregates by encapsulating and degrading them. Autophagy is also required for maintaining a healthy mitochondrial network, presumably by eliminating old and damaged mitochondria (17, 18). The importance of this process is shown by the accumulation of swollen and defective mitochondria in hepatocytes and MEFs from mice lacking the key autophagy gene *Ulk1* (17) and the appearance of deformed mitochondria in hepatic cells in *Atg7*-deficient mice (18).

The autophagic elimination of mitochondria, mitophagy, appears to be intimately linked to mitochondrial fission and fusion processes. A study of fibroblast mitochondrial dynamics showed that one in five daughter mitochondria is depolarized and eliminated by mitophagy (19). In most fission events, one daughter mitochondrion is transiently hyperpolarized while the sister mitochondrion is hypopolarized, suggesting that fission embodies a “stress

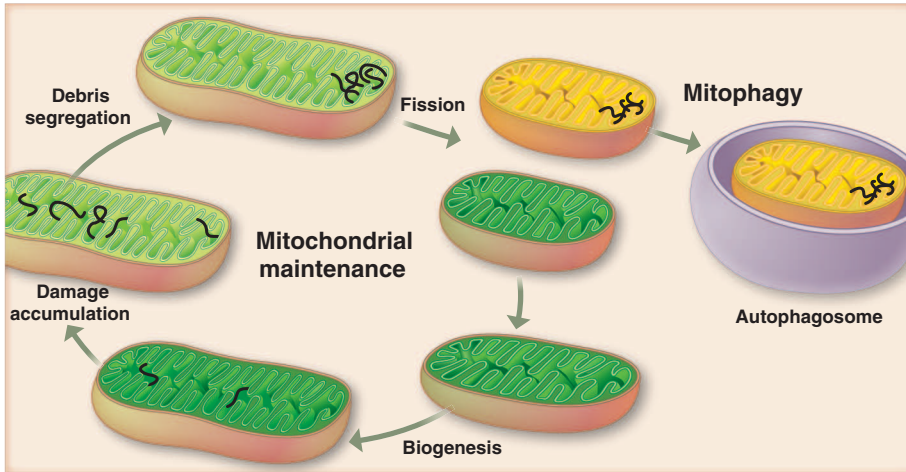


Fig. 2. Autophagy could purify the cellular pool of mitochondria if debris is aggregated and segregated by fission in a subset of mitochondria. If deleterious components (black fibers) are asymmetrically distributed or aggregated, fission could lead to cleansing of daughter mitochondrion (green) by preventing fusion and inducing mitophagy of the impaired ones (yellow).

substantia nigral neuron degeneration. PINK1- and Parkin-deficient *Drosophila* display muscle and neuron degeneration that is associated with swollen and defective mitochondria (25–27). Consistent with the model that mitochondrial fission and fusion promotes mitochondrial quality control, inhibition of mitochondrial fusion or promotion of mitochondrial fission compensates for deficiencies of PINK1 and Parkin in flies. Furthermore, Parkin overexpression in flies rescues unfolded protein stress of mitochondria through autophagy (28), and stimulation of autophagy rescues depolarized mitochondria accumulation in dopaminergic neurons from Parkin-deficient *Drosophila* (29).

Banish Mitochondria That Truly Are Uncoupled

Defective mitochondria can be toxic by generating excessive amounts of ROS, by consuming ATP through reversal of ATP synthase, and by interfering with a host of other metabolic processes (Box 1). Low levels of damage might be corrected by complementation through mitochondrial fusion, but badly damaged mitochondria will contaminate other mitochondria if they are allowed to rejoin the mitochondrial network before their elimination by autophagy. Several mechanisms are at work to stop this from happening. A first line of defense is provided by a built-in requirement of the mitochondrial inner membrane fusion machinery for membrane potential (30). Vertebrates have elaborated on this mechanism by providing a second line of defense through proteolytic inactivation of the inner membrane fusion dynamin OPA1. Proteolysis is mediated by the mitochondrial inner membrane protease OMA1, which is rapidly activated by low membrane potential and low levels of ATP (31, 32). The outer membranes of these mitochondria can still fuse, even without functional OPA1 or membrane potential, but the inner membrane-bound matrix compartments do not fuse, resulting

in several matrix compartments surrounded by a common outer membrane, like peas in a pod.

The last line of defense is provided by the Pink1 and Parkin pathway through the ubiquitination of the mitochondrial outer membrane fusion proteins Mfn1 and Mfn2. Ubiquitination of these proteins leads to their extraction from the membrane by p97 and their degradation by proteasomes (16). In addition, Pink1 and Parkin disrupt mitochondrial motility by degrading the small GTPase Miro, which serves as an adaptor for kinesin-dependent transport

and is also needed for mitochondrial fusion (33). Ultimately, uncoupled mitochondria lose both their inner and outer membrane fusion machineries, thereby preventing them from fusing with and poisoning the healthy mitochondrial network. Purposeful segregation and disposal of damaged mitochondria through changes in fission and fusion pathways are therefore integral parts of mitochondrial quality-control mechanisms.

Is Debris Also Sorted Inside Mitochondria?

The gradual accumulation of damaged components poses a problem for the mitophagic disposal process. If damaged components were evenly distributed, then the simple act of fission through Drp1 would not generate the asymmetry needed for inducing mitophagy by selective loss of membrane potential. It seems that asymmetric sorting of debris would be needed to generate the differences in membrane potential between daughter mitochondria that have been observed immediately after fission (19). Accumulation of damaged components in a subset of daughter mitochondria would enable their selective disposal, thus helping to rejuvenate the remaining population of mitochondria (Fig. 2).

How might mitochondria achieve this type of asymmetric fission? The mechanism is not yet known, but it seems likely that damaged proteins form aggregates within the mitochondrial matrix. Perhaps there is a way to stow these aggregates at the tips of mitochondria, thus providing a starting point for polarized fission. A precedent for this was set by bacteria, which remove aggregates by

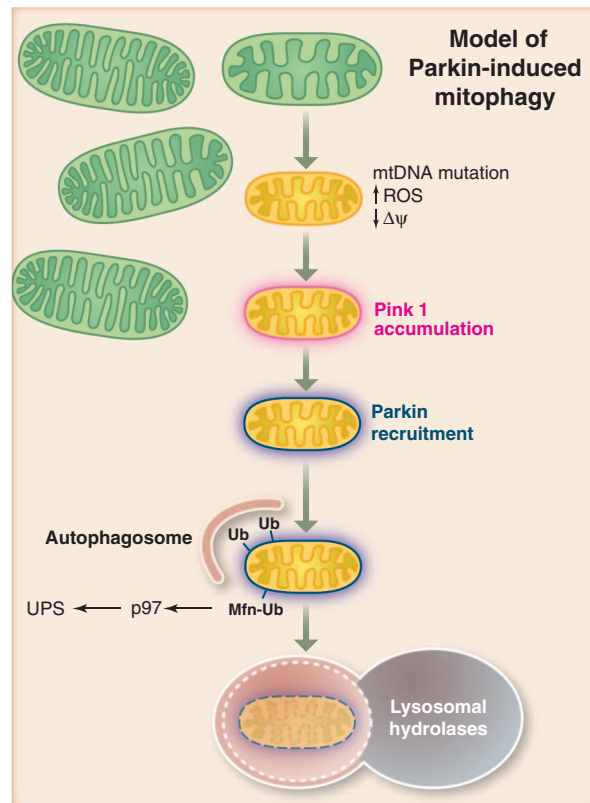


Fig. 3. PINK1 is constitutively degraded by the inner mitochondrial membrane protease PARL and maintained at low levels on healthy mitochondria. When a mitochondrion becomes damaged to the point of depolarizing the membrane potential across the inner membrane, PINK1 import to the inner membrane is prevented, thereby sequestering it on the outer mitochondrial membrane and away from PARL. PINK1 accumulates there and recruits the E3 ligase Parkin from the cytosol via PINK1 kinase activity. Parkin conjugates ubiquitin (Ub) to a variety of proteins on the outer mitochondrial membrane and mediates the proteasomal elimination of mitofusins 1 and 2. Lastly, Parkin induces autophagic elimination of the dysfunctional mitochondria. This pathway may constitute a quality-control mechanism to eliminate damaged mitochondria. UPS, ubiquitin proteasome system.

asymmetric fission, thus enhancing the growth rates of those daughter cells that do not receive aggregates (34). A similar asymmetry was observed during mammalian cell division, where aggregates accumulate at the centrosome and are selectively inherited by one of the two daughter cells (35). If mitochondria also have such a deliberate mechanism, then they might have a mechanism for inducing fission when too many aggregates are formed inside mitochondria.

Such an inducing mechanism is suggested by genetic studies showing that Pink1 and Parkin act upstream of the fission machinery in *Drosophila*. However, studies with mammalian cells have only shown effects of Pink1 and Parkin after fission is completed. Mammalian cells may have developed an additional, as yet undiscovered, mechanism to induce fission when mitochondrial aggregates accumulate, analogous to the rapid proteolytic inactivation of the fusion machinery through Oml-mediated proteolysis when mitochondria lose membrane potential or ATP.

Aggregation of misfolded proteins in the cytosol is facilitated by p62 and NBR1, which can lead to their disposal by autophagy (36). Interestingly, p62 also accumulates on mitochondria after Pink1 and Parkin activation. Once there, p62 triggers mitochondrial aggregation through its oligomerization domain (36). Mitochondrial aggregation may be an indirect result of aggregating ubiquitinated proteins on the mitochondrial outer membrane to segregate debris before fission. When protein damage accumulates, small vesicles bud from the outer mitochondrial surface. The trafficking of these vesicles to lysosomes suggests another and surprisingly direct pathway of mitochondrial debris removal that is independent of Drp1, therefore independent of classic mitochondrial fission, and also independent of autophagy (37).

Selective Removal of Mutant mtDNA

Can mitophagy cleanse genotoxic stress in addition to proteotoxic stress? Mutations in mtDNA accumulate as mammals age and could accumulate generation after generation were it not for germline purification of mtDNA. Although the mechanisms are not yet known, mitochondrial genomes with strong deleterious mutations can be removed during oogenesis (38, 39). Models for this cleansing mechanism include selective expansion of less impaired mitochondria to populate oocytes, apoptosis of oocytes with excessive mutant mtDNAs, and removal of poorly functioning mitochondria by mitophagy. Whether mutated mtDNA is selectively removed from somatic cells is not known.

A requisite for elimination of deleterious mitochondrial DNA mutations by mitophagy, be it in the germ line or soma, is physical linkage between the mutated mtDNA and the mutated gene product (40). Might there be a mechanism to identify malfunctioning nucleoids through their defective protein products, for example, through physical association with protein aggregates? Integral inner membrane proteins diffuse much more slowly than soluble matrix or intermembrane space proteins and there-

fore are more likely to be retained with their parental nucleoid than soluble tRNAs after mitochondrial fusion and fission events. This physical proximity might link nucleoids with mutant gene products that affect protein coding sequences and facilitate their autophagic purification. Such differential diffusibility between integral membrane proteins and tRNAs might explain why mutations in tRNAs are much more common in human diseases than mutations in the integral protein components of the oxidative phosphorylation machinery. Also, protein aggregates may start to form immediately during protein synthesis, which is physically linked with nucleoids (41). A mechanism for purifying mtDNA, by retaining mutant proteins with their genome, may prove to be an unexpected bonus of coupled transcription and translation to mitochondrial nucleoids.

Mitochondrial Fission and Apoptosis

When all else fails, stressed cells undergo apoptosis. In the past decade, many connections have been discovered between apoptosis and mitochondrial dynamics, as discussed more fully in this issue by Hoppins and Nunnari. High levels of cell stress that lead to apoptosis also lead to excessive fission of mitochondria. This occurs almost simultaneously with two steps of apoptosis that involve mitochondria: translocation from the cytosol to mitochondria of the pro-apoptotic Bcl-2 family member Bax and cytochrome c release. When Bax translocates to mitochondria, it accumulates in concentrated foci that colocalize with Drp1 and mitofusins. Inhibition of mitochondrial fission by Drp1 knock-down delays cytochrome c release, indicating that mitochondrial fission participates in Bax-mediated permeabilization of the outer mitochondrial membrane (42). The link may be that Bax is activated to oligomerize and release cytochrome c by membrane hemifusion intermediates that are formed during mitochondrial fission (43). Intriguingly, Bcl-2 family members also participate in mitochondrial fission and fusion in nonapoptotic cells (44). Thus, mitochondrial dynamics are involved not only in regulating individual mitochondrial fidelity within cells but also at the whole-cell level by participating in apoptotic cell death.

Outlook

Fusion allows mitochondria to compensate for one another's defects by sharing components and thereby helps maintain energy output in the face of stress. However, when a certain threshold of damage is reached, mitochondria are eliminated wholesale by autophagy. Fission segregates the most seriously damaged mitochondria to preserve the health of the mitochondrial network in addition to regulating morphology and facilitating mitochondrial trafficking. The highly dynamic mitochondrial fusion and fission cycle is proposed to balance two competing processes: compensation of damage by fusion and elimination of damage by fission. Failure of these stress responses may lead to neuron death and neurodegenerative disorders. In-depth understanding of mitophagic processes could aid the development of new treatments for mito-

chondrial and neurodegenerative diseases: It was recently shown that reactivation of autophagy can mitigate certain other diseases, such as muscular dystrophies associated with mitophagy (45).

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Acknowledgments: We thank members of the Youle lab for thoughtful comments. This work was supported by Intramural Program of the National Institute of Neurological Disorders and Stroke and grants from the NIH (GM051866) and the NSF (0552271) to A.M.v.d.B.

10.1126/science.1219855

Interception of Excited Vibrational Quantum States by O₂ in Atmospheric Association Reactions

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Bimolecular reactions in Earth's atmosphere are generally assumed to proceed between reactants whose internal quantum states are fully thermally relaxed. Here, we highlight a dramatic role for vibrationally excited bimolecular reactants in the oxidation of acetylene. The reaction proceeds by preliminary adduct formation between the alkyne and OH radical, with subsequent O₂ addition. Using a detailed theoretical model, we show that the product-branching ratio is determined by the excited vibrational quantum-state distribution of the adduct at the moment it reacts with O₂. Experimentally, we found that under the simulated atmospheric conditions O₂ intercepts ~25% of the excited adducts before their vibrational quantum states have fully relaxed. Analogous interception of excited-state radicals by O₂ is likely common to a range of atmospheric reactions that proceed through peroxy complexes.

Much of our microscopic understanding of chemical reactions derives from experimental and theoretical studies of small molecules in the gas phase, in which it has been known for some time that bimolecular reactions can yield products with vibrationally excited quantum states. For example, going back to Polanyi (1) a number of studies have shown that atom + diatom reactions have an energy distribution in the products that is sensitive to the position of the transition state, with early barrier processes favoring vibrationally excited products and late barrier processes favoring translational excitation. Similarly, several studies have shown that the kinetics of gas phase association reactions depend on the relative efficiency with which vibrationally excited quantum states of the association adduct are quenched by bath molecules (2). Recent studies suggest that these small-molecule, gas-phase models extend to the chemical dynamics of larger polyatomic molecules in condensed phases, including common organic solvents (3–8).

Mostly because of experimental complexity, a less understood aspect of chemical reactivity is the effect that reactant vibrational excitation has on association processes—that is, for reactions of type A* + B. A few experimental studies have shown that mode-selective reactant excitation affects the outcomes of chemical reactions under single-collision conditions (9–12). For example, Crim and co-workers famously examined the

H + HOD reaction and showed that 4 quanta of initial excitation in the reactant H–OD stretch led to H₂ + OD products, whereas 5 quanta in the reactant HO–D stretch led to HD + OH products.

For these sorts of single-collision, small-molecule studies, theoretical studies are increasingly able to provide complementary insight into the microscopic dynamics (13–17).

An open question is whether the chemical physics of mode-specific, single-collision A* + B studies carries over to common pressures and temperatures—for example, in Earth's atmosphere, combustion systems, biochemical systems, and common solvents. In these environments, molecules undergo fast energy exchange and randomization through bath interactions. Furthermore, individual chemical reactions are rarely isolated in such systems but coupled within larger kinetic networks. In this context, product excitation in one reaction may serve as reactant excitation for a subsequent reaction. Nevertheless, the almost universal assumption in describing association reactions under such conditions is that reactants relax to equilibrium fast enough not to affect the subsequent chemistry (18). This considerably simplifies chemical modeling because it means thermal transition-state theory (TST) may be invoked to predict the chemical outcome of any given association process (19, 20).

In this study, we examine one of the prototypical reaction sequences within atmospheric oxidation chemistry and show that vibrational

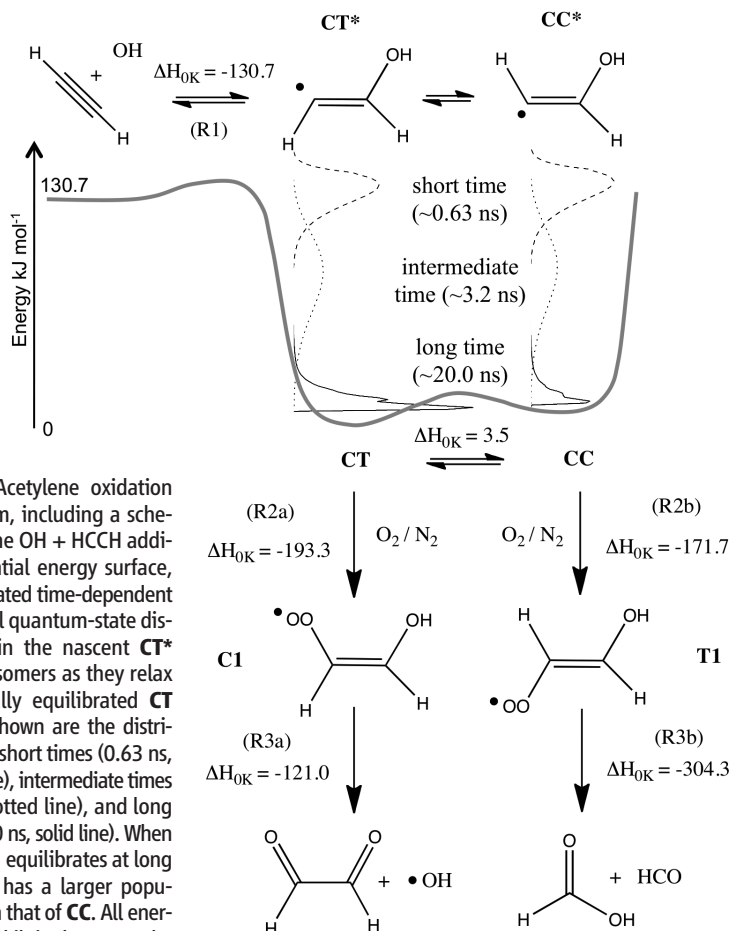


Fig. 1. Acetylene oxidation mechanism, including a schematic of the OH + HCCH addition reaction potential energy surface, and calculated time-dependent vibrational quantum-state distribution in the nascent CT* and CC* isomers as they relax to thermally equilibrated CT and CC. Shown are the distributions at short times (0.63 ns, dashed line), intermediate times (3.2 ns, dotted line), and long times (20.0 ns, solid line). When the system equilibrates at long times, CT has a larger population than that of CC. All energies are in kilojoules per mole. Reaction energies shown are taken from this work and previous work (21, 23).

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quantum-state excitation controls the product ratio of subsequent association reactions under atmospheric conditions. The target molecule is acetylene, which is prevalent in hydrocarbon combustion and an important atmospheric marker for pollution from road transport and biomass burning (21, 22). Atmospheric oxidation of acetylene begins with OH addition to make a beta-hydroxyvinyl radical adduct, which we refer to throughout this article as $\cdot$ BHV (more general OH radical adducts we refer to as $\cdot$ ROH). There are two important $\cdot$ BHV conformers, CT and CC in Fig. 1 [named to be consistent with (23)], that undergo rapid interconversion over a small forward barrier with a height of ~ 17 kJ mol $^{-1}$ (table S2). The kinetics of (R1) have been investigated in previous experimental and theoretical work (21, 23–27) and depend on the efficiency with which bath gas collisions quench vibrational excitation in the nascent $\cdot$ BHV adduct. Measurements of the (R1) rate coefficient (fig. S5) have a typical pressure fall-off (21, 27).

The manner in which $\cdot$ BHV oxidation proceeds is typical of unsaturated hydrocarbons and involves association with O $_2$ to make a peroxy radical, BHV-O $_2$ (23, 25, 28). Our calculations (fig. S2) show that O $_2$ addition to CT or CC is a barrierless process leading to either C1 or T1, both of which are shown in Fig. 1. Previously, Maranzana *et al.* showed that the lifetimes of C1 and T1 are very short at atmospheric temperatures and pressures (25, 26). This is because addition of O $_2$ to either CT or CC is effectively irreversible, with both channels having a downhill energy path to their respective dissociation products (26).

Hence, the O $_2$ addition step effectively determines the product identity: O $_2$ + CT gives prompt glyoxal + OH, and O $_2$ + CC gives prompt formic acid + HCO. The final product branching ratios consequently depend sensitively on the ratio of CT to CC. This in turn depends on the $\cdot$ BHV vibrational quantum-state distribution. At 298 K, the OH + HCCH association barrier is ~ 5.3 kJ mol $^{-1}$, resulting in an average inter-

nal energy for the nascent CT* and CC* of ~ 146.6 kJ mol $^{-1}$ (Fig. 1, short-time dashed line). At these energies, the state densities of CC* and CT* are comparable, giving a population ratio of $\sim 50:50$. At 298 K thermal equilibrium (Fig. 1, long-time solid curve), the situation is rather different, and the CT:CC population ratio is $\sim 78:22$.

The critical quantity is the extent of vibrational state excitation in CT* and CC* at the moment of O $_2$ addition. If we imagine conditions of pure O $_2$ in which every single collision with O $_2$ produced the corresponding peroxy radical (C1 or T1), then the final product ratio would be equal, corresponding to the integrated ratios of the short-time dashed lines in Fig. 1. However, some vibrational state quenching is inevitable, resulting in the intermediate-time quantum-state distribution (dotted line) shown in Fig. 1. As $\cdot$ BHV quenching increases, we would expect an increase in the OH yield because the energy-dependent equilibrium favors CT.

Experimentally, we tuned the proportion of vibrationally excited $\cdot$ BHV by varying the fraction of O $_2$ in N $_2$ and measuring the corresponding OH yield from (R3a). N $_2$ undergoes nonreactive collisions with $\cdot$ BHV that quench nascent vibrational excitation with a collisional efficiency similar to O $_2$. We conducted our experiments using a flash photolysis–laser-induced fluorescence (FP-LIF) apparatus (materials and methods and scheme S1, supplementary materials) (29). This setup uses two laser pulses: The first photolytically generates OH radicals, and the second probes the OH radical concentration after some time delay. By varying the time delay between the photolysis and probe pulses, it is possible to build time-dependent OH decay traces (fig. S1) at a range of pressures, temperatures, and N $_2$ /O $_2$ ratios. As OH reacts with acetylene via (R1), its concentration decays; however, production of OH via (R2a) and (R3a) results in a slower effective OH decay, and a kinetic analysis of the sort detailed in the supplementary materials (section 1) permits us to work out the effective OH yield from this channel. Our experiments substantially extend the range of conditions examined in previous experimental work, which identified an OH production channel at room temperature (24, 30).

The measured 298 K OH yield from (R3a) as a function of both the total bath gas pressure (O $_2$ and N $_2$) and the O $_2$ fraction (f_{O_2}) is shown in Fig. 2A. The results show that the OH yield is sensitive to f_{O_2} , as noted by Bohn *et al.* (24). For an O $_2$ /N $_2$ mixture with $f_{O_2} \sim 0.01$, the OH yield is 0.79 ± 0.02 (where the errors are statistical at the 2 σ level), which is equal to the fraction of CT at thermal equilibrium. With increasing f_{O_2} , O $_2$ intercepts an increasing proportion of CT* and CC* before relaxation, and the OH yield decreases. An f_{O_2} of ~ 0.88 gives an OH yield of 0.61 ± 0.02 . Over the experimental pressure range of 10 to 75 torr, the OH yield is insensitive to the total pressure (Fig. 2A). Calculations

Fig. 2. Experimental OH yields within a bath gas, M, consisting of a mixture of N $_2$ and O $_2$ as a function of (A) O $_2$ fraction, f_{O_2} , at a total pressure of 10, 25, and 75 torr, and (B) temperature, at average f_{O_2} of 0.013, 0.19, and 0.88. The lines show the results obtained from the corresponding ME/VRC-TST calculations. The ME/VRC-TST lines (corresponding to 10, 25, and 75 torr) in (A) are indistinguishable. Statistical error bars at the 2 σ level are shown.

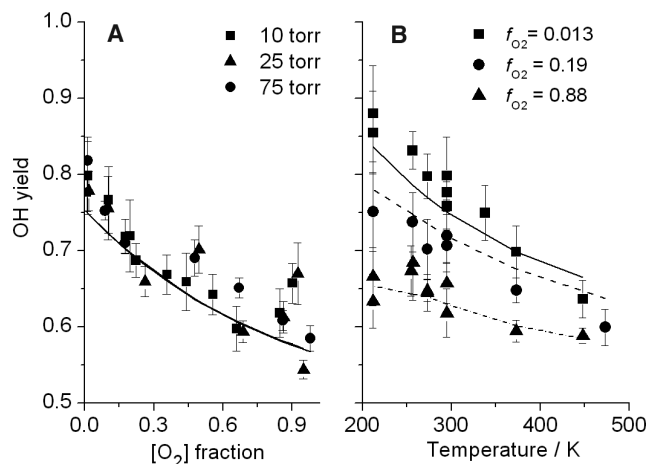
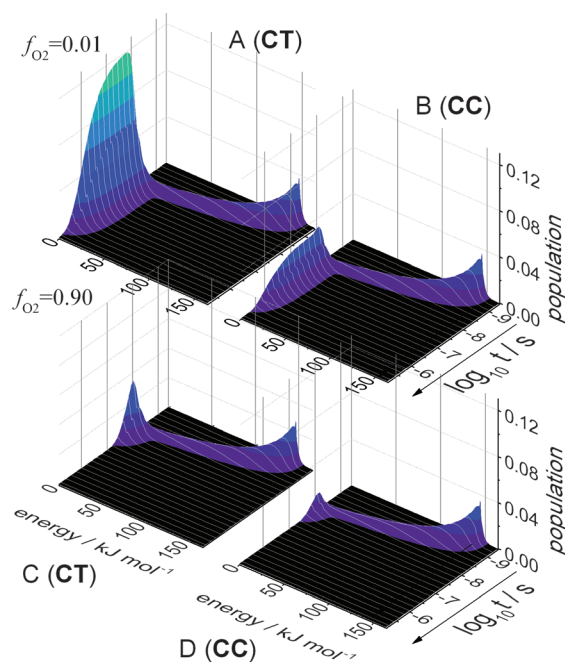


Fig. 3. Results from ME/VRC-TST calculations at 298 K and 760 torr showing evolution of the vibrational-state distributions in CT and CC as a function of both energy and time. (A and B) Results with $f_{O_2} = 0.01$ for CT and CC, respectively. (C and D) Results with $f_{O_2} = 0.9$ for CT and CC, respectively.



described below and in the supplementary materials, sections 2 to 4, substantiate this finding: The OH yield depends only on the ratio of reactive to nonreactive collisions, irrespective of pressure. At atmospheric f_{O_2} (0.2), there is an OH yield of 0.71 ± 0.03 (Fig. 2A), indicating that CT and CC are not in thermal equilibrium and that O_2 intercepts excited vibrational quantum states of $\cdot$ BHV before full relaxation.

The temperature dependence of the OH yields (212 to 473 K) at f_{O_2} values of 0.013, 0.19, and 0.88 are shown in Fig. 2B, presenting further evidence for O_2 intercepting vibrationally excited $\cdot$ BHV. At $f_{O_2} \sim 0.013$, most $\cdot$ BHV collisions are with N_2 and thus nonreactive. In this regime, the OH yield essentially corresponds to the temperature-dependent fraction of CT at thermal equilibrium. At $f_{O_2} \sim 0.88$, the probability of $\cdot$ BHV being intercepted by O_2 before quantum-state relaxation is highest, resulting in low OH yields over the entire temperature range. The small temperature dependence at $f_{O_2} \sim 0.88$ indicates that the product ratio is less sensitive to the thermal populations of CC and CT.

At any given f_{O_2} , the observed temperature-dependence of the OH yield reflects the initial quantum-state distribution at short times. At high temperatures, the short-time quantum-state distribution shown in Fig. 1 has a high-energy tail. Hence, the Fig. 2B data above 450 K show the lowest OH yields, and the data at 212 K show the largest OH yields. Under atmospheric conditions (298 K and $f_{O_2} \sim 0.2$), quantum-state relaxation is clearly in an intermediate regime, with a substantial fraction of the $\cdot$ BHV ensemble intercepted by O_2 before full relaxation of the vibrational quantum distribution to thermal equilibrium.

To put our mechanistic interpretation of the results shown in Fig. 2 on firmer quantitative footing, we formulated a model that combines ab initio quantum chemistry with stochastic master equation (ME) simulations (23, 31, 32) and variable reaction coordinate TST (VRC-TST) (33). Using this approach, we set up a coupled three-state system, including $OH + HCCH$, CT, and

CC. The state space of CT and CC was partitioned into energy grains, which represent combinations of excited rotational and vibrational quantum states at a particular energy. Within the energy-resolved state space, the CC and CT isomers could undergo (i) interconversion to the complementary $\cdot$ BHV isomer, (ii) upward and downward energy transfer arising from inelastic N_2/O_2 collisions, (iii) redissociation back to $OH + HCCH$, and (iv) reactive collisions with O_2 to produce either C1 or T1 with radical-radical association rate coefficients calculated by using VRC-TST (for CT + O_2 and CC + O_2 , the 298 K rate coefficients were 3.1×10^{-12} and 3.8×10^{-12} molecule $^{-1}$ cm 3 s $^{-1}$, respectively) (supplementary materials, sections 2 to 4).

The results of our ME/VRC-TST calculations at 298 K and 760 torr are shown in Fig. 3. The three-dimensional plots show the evolution of the CT and CC rovibrational quantum-state distributions shown in Fig. 1 are cuts taken from Fig. 3, A and B, at times that are short (0.63 ns), intermediate (3.20 ns), and long (20 ns), with respect to the $\cdot$ BHV relaxation time scales.

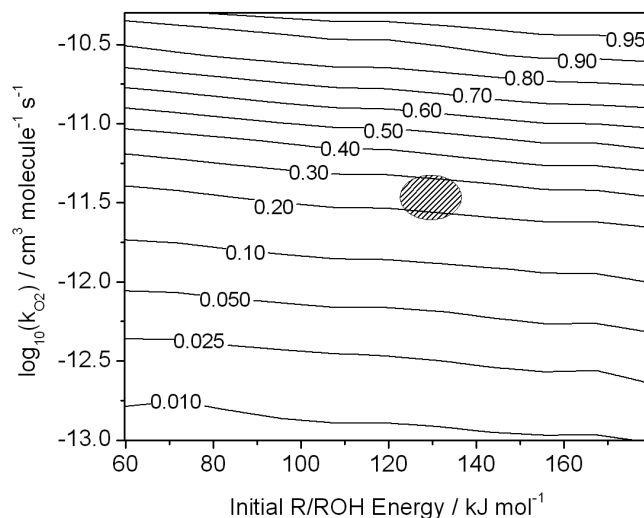
In Fig. 3, the total CT or CC population at some time t may be calculated by integrating over energy. For $f_{O_2} \sim 0.01$ (Fig. 3, A and B), the populations of CT and CC are essentially identical at times less than ~ 17 ns, which marks the approximate time at which vibrational deactivation is complete and thermal equilibrium sets in. By 17 ns, only 1.4% of the $\cdot$ BHV has been intercepted by O_2 . The bulk of O_2 addition takes place within the equilibrium regime over the next ~ 10 μ s, in which CT makes up $\sim 79\%$ of the total $\cdot$ BHV. When $f_{O_2} \sim 0.9$ (Fig. 3, C and D), the situation is rather different: $\sim 70\%$ of the $\cdot$ BHV is intercepted by O_2 before vibrational deactivation is complete at 17 ns, and $\cdot$ BHV depletion is complete within ~ 100 ns. At atmospheric f_{O_2} , the results lie between the limits shown in Fig. 3, with $\sim 25\%$ of the nascent $\cdot$ BHV intercepted by O_2 before the completion of vibrational deactivation, and $\cdot$ BHV fully depleted within ~ 400 ns.

The results discussed above provide strong evidence that the product-branching ratios for atmospheric acetylene oxidation are controlled by the fraction of vibrationally excited $\cdot$ BHV intercepted by O_2 before vibrational deactivation is complete. These results suggest a nontrivial role for $\cdot ROH^* + O_2$ and $\cdot R^* + O_2$ -type reactions under atmospheric conditions. Given that such association reactions are the dominant routes to atmospheric peroxy radicals, an open question is the extent to which the effects outlined in this paper occur in other oxidation systems (34). We investigated this question using the same sort of ME/VRC-TST model described above (supplementary materials, section 3). For a generic $\cdot ROH^*$ or $\cdot R^*$ radical, shown in Fig. 4 are the fraction of vibrationally excited radicals intercepted by O_2 as a function of (i) initial energy in the radical and (ii) the O_2 addition rate coefficient. For acetylene, in which $\sim 25\%$ of $\cdot$ BHV is intercepted by O_2 before vibrational quantum state relaxation, the relevant parameter space lies within the shaded circle.

For peroxy radical formation rate coefficients spanning 1×10^{-12} to 1×10^{-11} molecule $^{-1}$ cm 3 s $^{-1}$, O_2 intercepts a substantial fraction of radicals over a range of initial radical energies (Fig. 4). In constructing Fig. 4, we assumed that the energy transfer between arbitrary $\cdot ROH/R$ radicals and N_2/O_2 does not substantially differ from that which occurs between $\cdot$ BHV radicals and N_2/O_2 . A number of previous studies suggest that this assumption is reasonable (31), but further experimental and theoretical studies will be required to pin it down quantitatively and enhance our understanding of one of the most important reaction sequences in atmospheric chemistry.

Our results suggest that the sorts of effects highlighted in state-specific single-molecule conditions persist at atmospheric temperatures and pressures. Following recent work that has highlighted interesting dynamics in atmospherically relevant photochemical systems (35), our study shows that nonequilibrium effects carry over to non-photochemical systems. These cannot be explained using conventional thermal TST, and result in surprising effects on atmospheric reaction outcomes.

Fig. 4. Fraction of $\cdot R/\cdot ROH$ radicals intercepted by O_2 before vibrational-state relaxation, as a function of both the initial energy in the radical and the rate coefficient for O_2 addition. For the $\cdot$ BHV system investigated in this paper, the results lie within the shaded parameter space.



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Acknowledgments: D.R.G. is supported by Engineering and Physical Sciences Research Council Programme Grant

EP/G00224X. M.A.B., P.W.S., and J.L. received support from the Natural Environment Research Council via the National Centre for Atmospheric Science and grant NE/F018754/1. S.J.K. was supported by the U.S. Department of Energy, Office of Basic Energy Sciences, Division of Chemical Sciences, Geosciences, and Biosciences, under contract DE-AC02-06CH11357.

Supplementary Materials

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Materials and Methods

Figs. S1 to S6

Scheme 1

Tables S1 and S2

Sample MESMER Input File

References (36–58)

1 May 2012; accepted 11 July 2012
10.1126/science.1224106

Conduction of Ultracold Fermions Through a Mesoscopic Channel

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In a mesoscopic conductor, electric resistance is detected even if the device is defect-free. We engineered and studied a cold-atom analog of a mesoscopic conductor. It consists of a narrow channel connecting two macroscopic reservoirs of fermions that can be switched from ballistic to diffusive. We induced a current through the channel and found ohmic conduction, even when the channel is ballistic. We measured in situ the density variations resulting from the presence of a current and observed that density remains uniform and constant inside the ballistic channel. In contrast, for the diffusive case with disorder, we observed a density gradient extending through the channel. Our approach opens the way toward quantum simulation of mesoscopic devices with quantum gases.

The quantum simulation of models from solid-state physics using cold atoms has seen tremendous progress over the past decade (1, 2). Still, there are only limited analogies to the concept of conduction, which is at the core of mesoscopic solid-state physics. To close this gap, it would be highly desirable to connect a probing region in a cold-atom experiment to external incoherent reservoirs. This would lead to directed transport, the control of which is the basis of electronics. In such an intrinsically open configuration, boundary conditions play a crucial role, as in the Landauer theory of transport (3). The transport properties in cold atom systems have been investigated by observing the response of the system to variations of the external potential (4–13) or by monitoring the coherent evolution of bimodal Bose-Einstein condensates (14–16) as a response to a bias. Extending the concept of quantum simulation to conduction requires the engineering of macroscopic reservoirs, an atom battery or capacitor connected to the conductor (17–19).

We report on the observation of atomic conduction between two cold-atom reservoirs through a mesoscopic, multimode channel. Our measurement is made possible by the separation of scales

in our trap geometry (Fig. 1). The experimental configuration consists of two identical, macroscopic cold-atom reservoirs, which contain the majority of the atoms and feature fast equilibration dynamics. They are connected by a channel that contains a negligible fraction of the atoms and supports a few quantum states in the z direction, while it has the same extension as the reservoirs in the x direction, making it quasi-two-dimensional (quasi-2D).

We first prepared quantum degenerate gases containing $N_{\text{tot}} = 4 \times 10^4$ ^6Li atoms in each of the two lowest hyperfine states at a temperature of 0.36 ± 0.18 [$0.36(18)$] T_F , where $T_F \approx 700$ nK is the Fermi temperature in a combined optical and magnetic trap (20). A laser beam propagating along the x direction was focused on the center of the atomic cloud. The beam had a nodal line in the middle of its intensity profile and produced a repulsive potential for the atoms, which is tightly confining in the z direction (21, 22). Oscillation frequencies of up to 3.9 kHz along the z direction were achieved (Fig. 1).

Figure 2A presents a typical absorption picture of a cloud in the presence of the channel. We observed two clouds separated by a low density region, revealing the presence of the channel and confirming that it contains a negligible fraction of the total atom number (smaller than 0.01).

The conduction measurement proceeded as follows. We created an asymmetry in the potential by applying a constant magnetic field gra-

dient of 2.5 mT m^{-1} along the y axis. This was done during the evaporation process and eventually resulted in an imbalance $\Delta N/N_{\text{tot}} \approx 0.2$, where ΔN is the number difference between right and left reservoirs. After evaporation, the confining potential of the trap was increased, and a uniform magnetic field was set to 47.5 mT. At this value, the scattering length of atoms in the two internal states is $-100 a_0$, with a_0 being the Bohr radius. This ensures that the collision rate is sufficient to maintain thermal equilibrium in each reservoir on a time scale of ~ 30 ms. It also ensures that the mean free path (~ 1.3 mm) is much larger than the length of the channel, making it ballistic. The symmetry of the trapping potentials was then restored by switching off the magnetic field gradient in 50 ms, a time longer than the internal thermalization time of each reservoir but short compared with the time scale of equilibration of the populations of the two reservoirs. Figure 2B shows the difference between an absorption picture taken with and without imposing an imbalance. The right reservoir is seen to contain an excess of particles

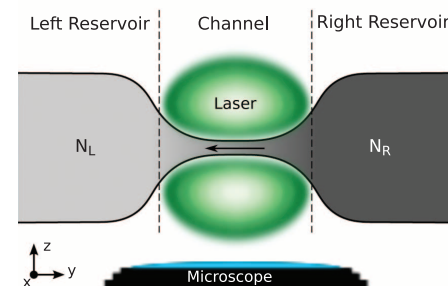


Fig. 1. Experimental configuration. A macroscopic ^6Li cloud was divided into two reservoirs separated by a narrow channel. The channel was imprinted by using the two lobes in the intensity profiles of a nearly TEM_{01} -mode laser beam at the wavelength of 532 nm, created with a holographic plate. The distance between the two lobes is $18 \mu\text{m}$, and the waist of the beam in the y direction is $30 \mu\text{m}$. A microscope objective [numerical aperture (NA) = 0.55] was used to observe or manipulate the atoms in the channel. When N_R is larger than N_L , an atomic current I flows through the channel (arrow).

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compared with the balanced reservoirs situation, and the left reservoir shows a deficit of particles.

The equilibrium of the whole system is characterized by a balanced population of both reservoirs; thus, after restoring the symmetry of the trap, an atomic current sets in through the channel. Figure 3A presents the time evolution of $\Delta N/N_{\text{tot}}$ with the oscillation frequency along z in the channel set to 3.9 kHz. We observed an exponential decay (Fig. 3A, solid line), with a time constant of 170 ± 14 [170(14)] ms. This exponential shape suggests a direct analogy with the discharge of a capacitor through a resistance. Indeed, the evolution of the system can be described as

$$\frac{d}{dt} \Delta N = -\frac{G}{C} \Delta N \quad (1)$$

where G is the conductance of the channel, $C = \partial N / \partial \mu$ is the compressibility of the reservoirs, and μ is their chemical potential. The compressibility is analogous to the capacity of a capacitor. We neglected possible thermoelectric effects, because we did not observe a noticeable temperature evolution in the reservoirs.

Because the decay is the slowest process, we have a quasi-steady-state situation at each point in time. Thus, the derivative of the curve around any point is a measurement of the current at a certain number difference, where the atoms in each reservoir have a known, thermal distribution. Therefore, the magnitude of the current measures the dc characteristic of the channel. Figure 3B shows the observed current as function of the number difference for the same data set (circles) and for a channel with reduced confinement of 3.2 kHz at the center (triangles). A linear relation is manifest for both cases, which confirms dissipative, ohmic conduction and allows us to extract the slopes $G/C = 2.9(4) \text{ s}^{-1}$ and $3.7(2) \text{ s}^{-1}$, respectively.

The observation of resistance in the ballistic conduction shows that the boundary conditions are essential in the investigation of transport, as in the Landauer approach. Indeed, the free ex-

pansion of a noninteracting cloud is also ballistic, but the absence of connection to reservoirs leads to the absence of any resistance to the flow, other than inertia. Furthermore this ballistic expansion generally does not depend on the conduction properties of the initial cloud, because ballistic expansion has even been observed for a band insulator (12).

The Landauer-Büttiker formula states that, at zero temperature, the conductance of a ballistic conductor is equal to $1/h$ per quantum state contributing to the conduction, where h is Planck's constant (23). Because of the quasi-2D character of the channel, the current is carried by many transverse modes, which are not individually resolved because of finite temperature. Instead, as the channel confinement is varied, the resistance is expected to vary linearly with the oscillation frequency along the confined direction z , because of the variations in the number of available modes. In both measurements, the reservoirs have the same compressibilities; thus, the ratio 0.76(11) of the two slopes is equal to the ratio of conductances alone and agrees qualitatively with the inverse ratio 0.82 of trap frequencies along z . We found that the linear relation between resistance and trap frequency persists for various confinements within the accessible range. The contact resistance, which naturally appears in the Landauer picture, explains the observation of ohmic conduction even in a defect-free channel. Although every atom that enters the channel on one side exits on the other with the same momentum with probability one, only a tiny fraction of the atoms from each reservoir can pass through the channel at any given time because of the low density of states in the channel (24, 25).

To gain further insight into this mechanism, we used high-resolution microscopy to observe the density distribution of atoms in the channel. We did so by using in situ absorption imaging along the z direction, with and without current flowing through the channel. A typical picture of the density distribution in the channel in the absence of

current is presented in Fig. 4A. At the sides of the picture, we observe the contacts with the two reservoirs that extend beyond the field of view. Closer to the center, the lower column density reveals the presence of the channel, which is smoothly connected to the reservoirs. Figure 4B shows the difference between two such pictures, taken with and without current flowing through the channel. We see the small density difference between the two reservoirs, which reflects the macroscopic number difference shown in Fig. 2B.

The red points in Fig. 4C show the line-density difference $\sim n_l$ along the channel, obtained by accumulating the image in Fig. 4B along the x direction. At the center of the channel, the difference is close to zero over a length of 30 μm , whereas the density difference changes quickly at the sides of the channel. This qualitative difference between the center of the channel and the sides indicates that the resistance observed in Fig. 3 originates from the reflection of atoms by the contacts with the reservoirs (26).

As opposed to the ballistic channel, we have engineered a channel where the conduction is diffusive, which is the case encountered in typical solid materials. To do so, we projected a blue-detuned laser speckle pattern onto the channel, realizing a quasi-2D disorder (27). This pattern has a gaussian envelope with a root mean square diameter of 32 μm , an average amplitude of 0.6 μK at the center, and a correlation radius of 0.37 μm (28). We then reduced the confinement of the channel down to 1.6 kHz along z , so that the atomic conductance of the disordered channel was the same as that of the ballistic one studied before. We thus have a second system displaying the same macroscopic transport properties but with a different conduction mechanism. The measured line-density difference in the disordered channel is shown in blue in Fig. 4C (blue dots). In sharp contrast to the ballistic case, the density difference exhibits a continuous decrease from right to left.

For the diffusive transport case, we now relate the variations of density difference to local

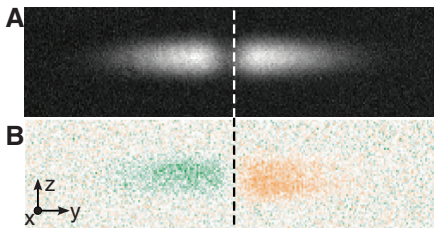
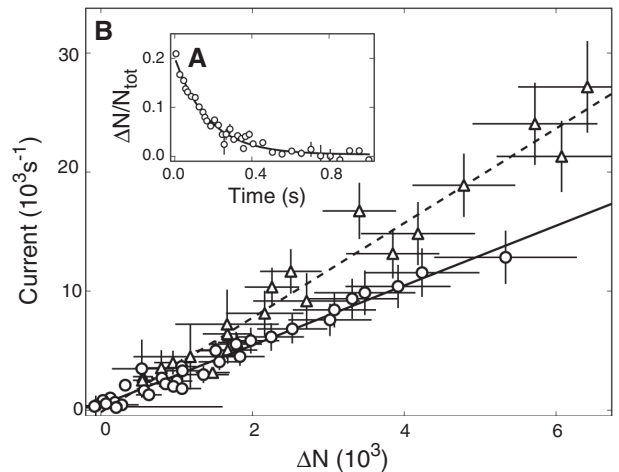


Fig. 2. Atomic reservoirs connected by a mesoscopic channel. (A) Absorption image of the atoms before the imbalance is applied. The image was taken after a 1-ms expansion in the x - z plane. The dark region at the center reveals the presence of the mesoscopic channel. The dashed line tracks the position of the channel on both panels. (B) Density difference between the cloud in the unbalanced configuration, as the current sets in, and the equilibrated cloud. Excess of atoms is displayed in orange, and lack of atoms is displayed in green. The imbalance of the reservoirs, $\Delta N/N_{\text{tot}}$, was set to 0.2.

Fig. 3. Observation of ohmic conduction. (A) Measured number difference between the two reservoirs as a function of time. The solid line is an exponential fit to the data. (B) Current as a function of number difference between the two reservoirs, measured from the exponential fit of (A), for two different confinements in the channel. A small offset obtained from the fits in (A), which is due to a slight misalignment of the channel with respect to the center of the trap, has been subtracted. Circles, maximum center frequency along z set to 3.9 kHz; triangles, 3.2 kHz. The lines are linear fits to the data. Error bars indicate statistical errors.



transport quantities. Following the approach of mesoscopic physics (25), we introduced an effective local chemical potential, $\mu(y)$, by requiring that it yields the observed density when used in the Fermi-Dirac distribution. Because the mean free path for atomic collisions is very large, the energy distribution of atoms may not be thermal outside the reservoirs. Therefore, the definition of the effective chemical potential $\mu(y)$ does not correspond to the local density approximation and does not suppose local equilibrium.

From the current I measured across the channel, we deduce from Fig. 4C the local resistivity (28)

$$\rho = \frac{1}{\kappa_1 I} \frac{\partial \tilde{n}_1}{\partial y} \quad (2)$$

where we have introduced the line compressibility in equilibrium, $\kappa_1 = \partial n_1 / \partial \mu$, with n_1 being the line density along the y axis. The line compressibility was obtained directly from the column density at equilibrium and the shape of the trap (28, 29). Figure 4E presents the resistivity obtained by applying Eq. 2 to the in situ picture. It remains finite across the channel and presents two weak maxima, which we attribute to the fastest variations of the confining potential creating the channel. The local resistance in the channel has its origin in the scattering with the random potential, which leads to randomization of the momentum distribution of the atoms (25).

Many quantities of interest can be extracted from the microscopic density distribution. For instance, the drift velocity, $v_d = I/n_1$, in the channel is found to be $200 \mu\text{m s}^{-1}$, or $4 \times 10^{-3} v_F$, where v_F is the Fermi velocity in the reservoirs, which

confirms that our system realizes the Landauer paradigm of conduction. For the diffusive channel, we also introduced an atomic mobility for the atoms, $v_d \kappa (\partial n_1 / \partial y)^{-1}$, which relates the drift velocity to the effective chemical potential gradient and thus characterizes the intrinsic conduction properties of the channel regardless of the density. Figure 4F presents the atomic mobility as obtained from the in situ pictures for the diffusive channel, which remains finite through the channel, with a weak maximum at the center.

Our configuration is closely analogous to that of a field-effect transistor. The strength of the confinement in the channel has been used to vary the conductance by changing the density. Further tuning of the resistance could be obtained by adding a repulsive gate laser. In addition, the effects of disorder in such a device can be studied systematically by varying the laser-induced random potential. Metal-insulator transitions, such as two-dimensional Anderson localization (30), can be studied in a way that is directly analogous to real solid-state devices (31). The ability to further control the disorder could be used to study universal conductance fluctuations (3). Apart from disorder, various potentials can be designed and projected onto the channel using the microscope setup (20). This will allow us to measure the conduction properties of various model systems. For example, quantized conduction can be investigated if a single mode can be resolved in the channel (32–34). Furthermore, conductance is very sensitive to interactions between atoms and would be an ideal observable to investigate strongly correlated fermions. The combination

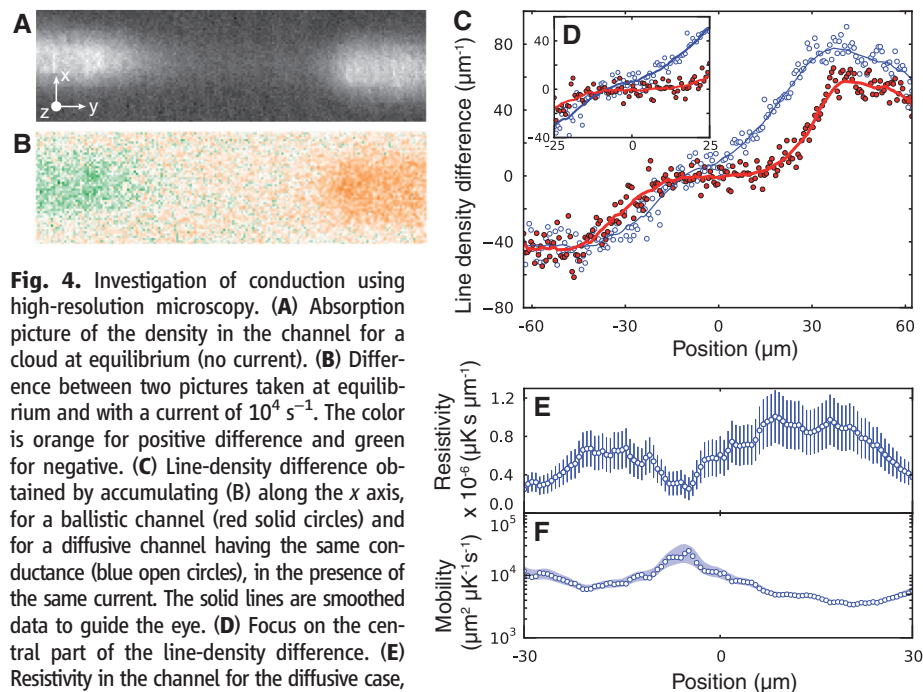


Fig. 4. Investigation of conduction using high-resolution microscopy. (A) Absorption picture of the density in the channel for a cloud at equilibrium (no current). (B) Difference between two pictures taken at equilibrium and with a current of 10^4 s^{-1} . The color is orange for positive difference and green for negative. (C) Line-density difference obtained by accumulating (B) along the x axis, for a ballistic channel (red solid circles) and for a diffusive channel having the same conductance (blue open circles), in the presence of the same current. The solid lines are smoothed data to guide the eye. (D) Focus on the central part of the line-density difference. (E) Resistivity in the channel for the diffusive case, as a function of position, computed from Eq. 2. Only the center of the channel is shown, where the extraction procedure for the line compressibility is valid (28). (F) Mobility in the channel as a function of position for diffusive channel. The error bars and shaded region reflect the uncorrelated combination of estimated systematic and statistical uncertainties.

of mesoscopic atomic devices with controlled interactions opens fascinating perspectives and could shine new light on open questions in the field of mesoscopic physics (35).

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Acknowledgments: We acknowledge fruitful discussions with J. Blatter, A. Georges, H. Moritz, A. Rosch, and W. Zwerger and the help of T. Müller during the early stage of the experiment. We are grateful to the group of F. Merkt for the loan of a 532-nm laser. We acknowledge financing from National Centers for Competence in Research Materials with Novel Electronic Properties (NCCR MaNEP) and Quantum Science and Technology (QSIT), European Research Council (ERC) project Synthetic Quantum Many-Body Systems (SQMS), Framework Program 7 (FP7) project Nanodesigning of Atomic and Molecular Quantum Matter, and ETH Zürich. J.P.B. acknowledges support from the European Union (EU) through a Marie Curie Fellowship.

Supplementary Materials

www.sciencemag.org/cgi/content/full/science.1223175/DC1
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Fig. S1

10 April 2012; accepted 16 July 2012
Published online 2 August 2012;
10.1126/science.1223175

Probing the Ultimate Limits of Plasmonic Enhancement

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Metals support surface plasmons at optical wavelengths and have the ability to localize light to subwavelength regions. The field enhancements that occur in these regions set the ultimate limitations on a wide range of nonlinear and quantum optical phenomena. We found that the dominant limiting factor is not the resistive loss of the metal, but rather the intrinsic nonlocality of its dielectric response. A semiclassical model of the electronic response of a metal places strict bounds on the ultimate field enhancement. To demonstrate the accuracy of this model, we studied optical scattering from gold nanoparticles spaced a few angstroms from a gold film. The bounds derived from the models and experiments impose limitations on all nanophotonic systems.

One of the most remarkable phenomena associated with metals at optical wavelengths is field enhancement. Local optical fields within a metal nanostructure can achieve strengths that are orders of magnitude greater than that of the incident field. This singular feature of metals serves as the fundamental mechanism for a host of radiative and scattering processes associated with nanophotonic systems. Field enhancement has been shown to affect surface-enhanced Raman scattering (1); nonlinear processes, such as enhanced harmonic generation (2) or wave mixing (3); nanolasing (4); plasmonic sensing (5); and enhancement of spontaneous emission (6).

The largest field enhancements in nanoplasmic systems occur near sharp asperities or corners associated with metal nanoparticles (NPs) and within the subnanometer gaps formed between NP aggregates. An incident optical field drives currents across the NP, resulting in peak currents flowing through the NP during one part of the cycle, and a peak surface charge density during the other part of the cycle. Using the conventional, classical description of the metal's response—or local model—at the moment of peak polarization, the charges can be considered crushed into a layer of infinitesimal thickness along the NP surface, resulting in the standard surface charge density picture. Structures that possess a singularity, such as spheres that touch at a point, have been shown to possess continuous scattering spectra associated with compression of the surface plasmon wave field at the singularity. According to the local model, a pulse of surface plasmons launched into such a system

would travel toward but never reach the singularity, giving rise to energy compression and enormous field enhancements (7).

It would appear, then, that virtually unbounded field enhancements should be possible if well-defined subnanometer gaps can be created between nanostructures with sufficiently smooth surfaces. However, in a real metal, polarization charge densities are not perfectly localized at a surface but are slightly spread over a thickness near the boundary. This dispersion of the charge effectively smoothes the singularities: Charges no longer reside exactly at the surface, but acquire some volume as the charge density spreads into the NP. The scattering spectrum ceases to be continuous and is now discrete, with correspondingly reduced field enhancements (8, 9). These effects have long been recognized by theorists; for example, Fuchs and Claro (10) showed that the nonlocal effects considered here limit the response of almost-touching spheres.

The local model for free electron response inside metallic structures is insufficient to describe metals whose critical dimensions are on the order of a few nanometers or less. A more appropriate description should take into account atomic and subatomic interactions, and

electron-electron repulsion in particular. The Pauli exclusion principle forbids two fermions from occupying the same quantum state at a given time, resulting in a repulsive force between charge carriers. Along with the classical Coulomb force, the quantum repulsion manifests itself as a pressure in an electron gas that resists the compression induced by an applied electromagnetic field. This electron pressure may be taken into account by a hydrodynamic description of the collective motion of the electrons inside a metal (11). The currents $\mathbf{J}$ inside a metal induced by an electric field $\mathbf{E}$ oscillating at frequency ω can be described by the following equation (12):

$$\beta^2 \nabla(\nabla \cdot \mathbf{J}) + (\omega^2 + i\gamma\omega)\mathbf{J} = i\omega\omega_p^2 \epsilon_0 \mathbf{E} \quad (1)$$

where ϵ_0 is the vacuum permittivity, and γ and ω_p are the damping coefficient and the plasma frequency, respectively, which also appear in the conventional Drude formula, $\epsilon(\omega) = 1 - [\omega_p^2/(\omega^2 + i\gamma\omega)]$, and β —approximately the speed of sound in the Fermi-degenerate plasma of conduction electrons (11)—is proportional to the Fermi velocity v_F .

The effect of including the pressure term in the electron response is that the longitudinal dielectric function, ϵ_L , becomes nonlocal, depending on the propagation vector $\mathbf{k}$ in addition to the frequency, as follows:

$$\epsilon_L(\mathbf{k}, \omega) = 1 - \frac{\omega_p^2}{\omega^2 + i\gamma\omega - \beta^2 |\mathbf{k}|^2} \quad (2)$$

whereas the transverse response is unchanged.

The simple picture, then, of a surface charge layer with infinitesimal extent must be replaced with a continuous charge density, whose extent will be determined by $\beta/\omega_p \propto \lambda_{TF}$, where $\lambda_{TF} = v_F/\omega_p$ is the Thomas-Fermi screening length. Rather than a strict surface charge density, the nonlocality produces a volume charge density that spreads out from the surface a distance $\sim \lambda_{TF}$ on the order of 1 Å. As a result, the real behavior

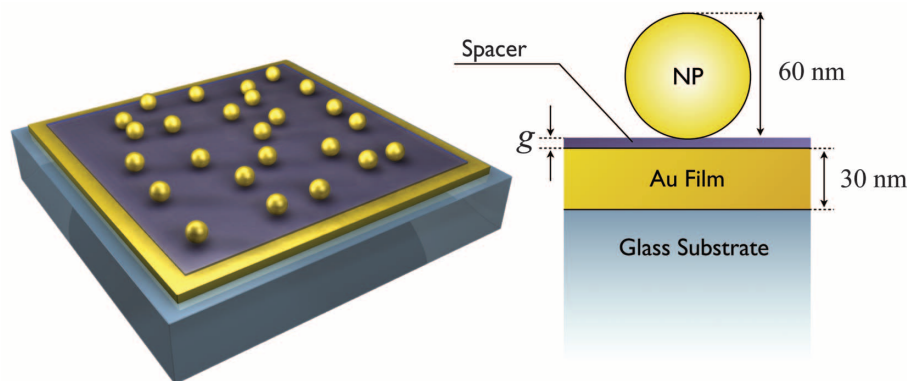


Fig. 1. Geometry of the film-coupled nanoparticle. **(Left)** Schematic of the sample. **(Right)** Cross section of a single film-coupled nanosphere.

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of the optical characteristics of subnanometer-length systems may deviate from local model predictions (13).

A full quantum treatment of optical response is possible only for very small spheres. Therefore, it is critical to develop and verify a semi-empirical model that can be applied to spheres of dimensions greater than a few nanometers. Here, we use the hydrodynamic model to take quantum effects into account, assuming that delocalization of surface charge is the dominant process. Alternative semi-empirical models have been developed that emphasize the tunneling current between two surfaces, which is present at very small separations (14–16). We find that for the geometrical parameters of our experiments, the hydrodynamic model gives an excellent account of our data, although we concede that tunneling current may well play an important role for smaller dimensions.

To date, the experimental study of nonlocality on coupled plasmonic systems has been ham-

pered by the difficulty in achieving reliable and precise control of subnanometer interparticle spacing. Even a relatively simple system, such as two nanospheres separated by a subnanometer gap, remains a challenge for colloidal or lithographic synthesis methods. By contrast, one closely related system—a metal nanosphere positioned a specified distance above a metallic film—is simple to fabricate and provides exquisite control of the spacing. The film-coupled nanosphere geometry (Fig. 1) involves the deposition of a metal film by standard sputtering or evaporation methodologies, followed by solution deposition of a molecular dielectric layer and chemisorption of chemically synthesized metal NPs on the spacer layer.

As the NPs are brought closer to the film, the coupling between a given NP and its virtual image induces a red shift in the peak of the plasmon resonance wavelength, which can be detected as the peak intensity in the measured scattering cross section. Because the spacer lay-

er exhibits tremendous uniformity, the scattering behavior of the NPs is remarkably uniform, and scattering measurements on a slide sample from ensembles of NPs are representative of the typical scattering of an individual film-coupled NP, as confirmed by dark-field microscopy. Numerical simulations reveal the expected behavior of strongly localized fields between the NP and film, related to the interaction of the NP with its electromagnetic image (Fig. 2). In addition, the field very near the surface of the metal sphere decays exponentially away from the surface on a scale given essentially by λ_{TF} .

The plasmon resonant scattering peak positions and enhancement factor for gap dimensions between 0.1 nm and 10 nm can be calculated using both the local model and the nonlocal model (Fig. 3). The plasmon resonance of the NP shifts predictably toward the red, and the field enhancement grows as the gap dimension decreases. If the local model for the metal dielectric function is used, the expected shift in

Fig. 2. Simulation of a single film-coupled nanoparticle. **(Left)** Relative electron surface density showing the excited surface plasmon polariton propagating over the metal film. The nanoparticle can be seen at the center. **(Upper right)** A plane wave is incident at 75° from normal on the nanoparticle. **(Lower right)** A close-up of the near fields surrounding the nanosphere; note the large field amplitude directly below the sphere. Looking closer yet, it can be seen that the fields penetrate into the nanosphere by a distance on the order of the Thomas-Fermi screening length.

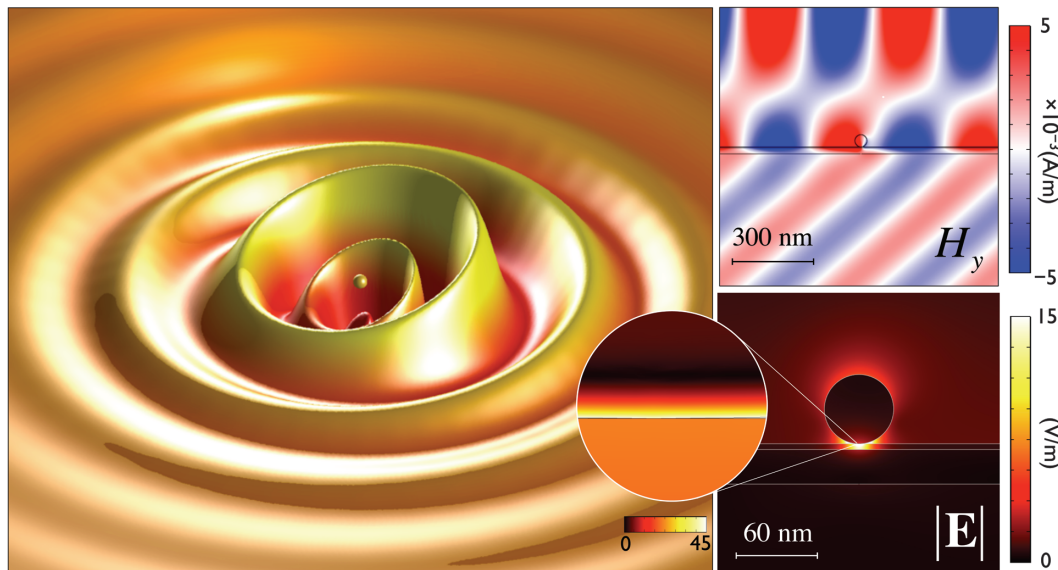
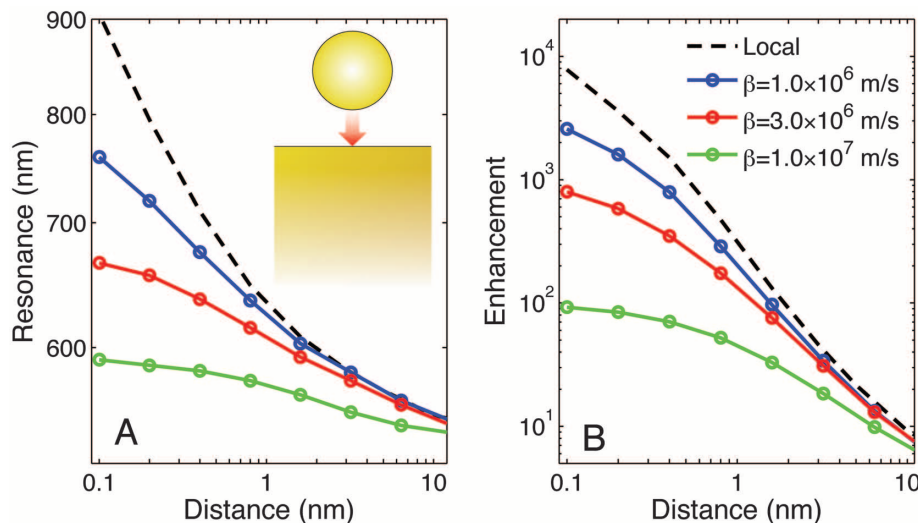


Fig. 3. Behavior of the film-coupled nanosphere, assuming a local model and the nonlocal model with various values of β , as a function of separation distance. Calculations refer to a gold nanosphere of radius $r = 30$ nm on a film 300 nm thick. **(A)** Position of the peak scattering intensity as a function of gap size. **(B)** The corresponding field enhancement ratio. Note that in the absence of nonlocal effects, the peak scattering wavelength is extreme and the field enhancement grows to enormous values; nonlocality places a limit on the ultimate enhancement.



the plasmon resonance wavelength is pushed to nearly $\lambda = 900$ nm, corresponding to a peak local field enhancement of $\sim 10^4$ (Fig. 3). The presence of a nonzero β considerably modifies the plasmon resonance wavelength shift for separation distances below 5 nm. From 1 nm to 0.1 nm, the impact of the nonlocal electronic response is decisive, causing the peak resonance wavelength to occur at values much lower than that predicted by the local model. For the realistic value of the nonlocal parameter, $\beta = 1.0 \times 10^6$ m/s—as expected from prior measurements and theory—the peak resonance wavelength shift is capped near 750 nm, a full 150-nm difference from that predicted using the local model.

The impact of spreading the charge thus leads to substantial optical shifts that are easily measurable by spectroscopic techniques. The field enhancement is still extremely large relative to the analogous 2D system (7), even with the nonlocal interactions taken into account. The expected enhancement exceeds values of 10^3 for realistic values of the parameter β (Fig. 3B). Far more than material losses, the nonlocality plays the dominant role in limiting electromagnetic enhancement of NPs, reducing the dimer or film-coupled NP peak enhancement by a factor of ~ 4 .

An experimental test of the validity of our predictions requires precise control over extremely short gap lengths. We deposit spacer layers using either layer-by-layer (LBL) deposition of

polyelectrolytes (5, 17, 18), for separations that range from 2.8 to 26.6 nm, or by the formation of self-assembled monolayers (SAMs) of amine-terminated alkanethiols for even smaller separation distances that range from 0.5 to 2.0 nm. We first prepare a set of gold films 30 nm thick, then incubate the gold films with either a series of polyelectrolytes or a set of amine-terminated alkanethiols wherein the gap length is tuned by the number of carbon atoms in the chain (Fig. 4A). The thicknesses of the SAM spacer layers have been estimated using a theoretical approach (12), as standard ellipsometry measurements have been shown to produce systematically low thickness values for such thin SAMs on gold surfaces (19).

The optical response of the NPs deposited on the spacer layers is measured by illuminating the sample with white light and collecting the scattered light through a dark-field objective. The collected light is then directed through an image plane aperture (diameter 1 mm) to the spectrometer. The plasmon resonant scattering spectra for each of the samples—which correspond to different gap sizes as determined by the chain length of the SAM—are shown in Fig. 4C. The results of both the local and nonlocal model simulations are plotted alongside the collected data in Fig. 4D, showing the plasmon resonance peak position dependence on film-NP separation distance. We found that the electric permittivity of the spacer layer must be taken into

account in the models to achieve the best fit. We used a nondispersive index of refraction of $n = 1.8$. Comparison of the numerical simulations to our experimental results (Fig. 4D) reveals that the nonlocal model is in excellent agreement with the experimentally measured scattering peaks, confirming that the actual dielectric function is modified by the electron pressure term.

The agreement obtained demonstrates that the hydrodynamic model is a powerful tool that incorporates quantum effects in macroscopic systems, and shows that in certain cases the impact of nonlocality may prevail over purely quantum effects such as electron tunneling. Although direct measurements of near-field enhancement remain difficult at such scales, our results provide strong experimental support in setting an upper limit to the maximum field enhancement achievable with plasmonic systems.

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Acknowledgments: We thank M. Scalora, S. Wolter, and A. Moreau for helpful input and discussion. Supported by Air Force Office of Scientific Research grant FA9550-09-1-0562 and by the Army Research Office through Multidisciplinary University Research Initiative grant W911NF-09-1-0539. Also supported by the Leverhulme Trust and the Marie Curie Actions (J.B.P., S.A.M., and A.I.F.-D.), NIH grant R21EB009862 (A.C.), and NIH F32 award F32EB009299 (R.T.H.).

Supplementary Materials

www.sciencemag.org/cgi/content/full/337/6098/1072/DC1
Materials and Methods
Supplementary Text
Figs. S1 and S2
References (20–25)

17 May 2012; accepted 18 July 2012
10.1126/science.1224823

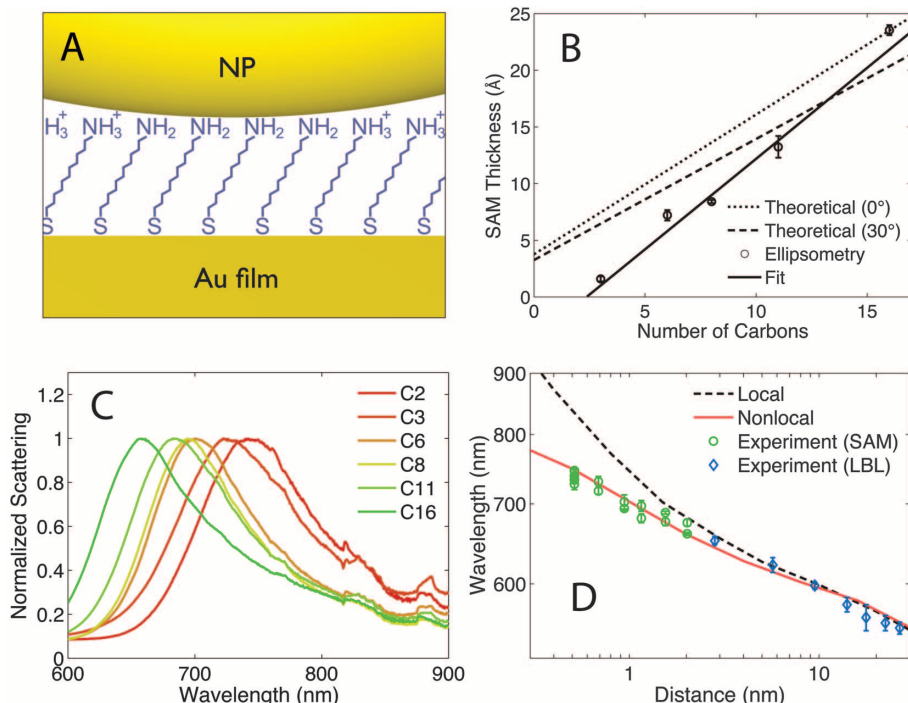


Fig. 4. Experimental confirmation of nonlocal contributions to surface plasmon scattering. (A) Schematic of nanoparticle-film gap system showing a gold nanoparticle separated from the film by an amine-terminated alkanethiol SAM. (B) Thickness of the SAM layers as a function of the number of carbon atoms. (C) Normalized dark-field measured spectra of ensembles of film-coupled nanoparticles for SAM spacer layers of different numbers of carbon atoms. (D) Comparison of experimental measurements from SAM- and LBL-type spacers with numerical results with $\beta = 1.27 \times 10^6$ m/s.

Biogenic Potassium Salt Particles as Seeds for Secondary Organic Aerosol in the Amazon

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The fine particles serving as cloud condensation nuclei in pristine Amazonian rainforest air consist mostly of secondary organic aerosol. Their origin is enigmatic, however, because new particle formation in the atmosphere is not observed. Here, we show that the growth of organic aerosol particles can be initiated by potassium-salt-rich particles emitted by biota in the rainforest. These particles act as seeds for the condensation of low- or semi-volatile organic compounds from the atmospheric gas phase or multiphase oxidation of isoprene and terpenes. Our findings suggest that the primary emission of biogenic salt particles directly influences the number concentration of cloud condensation nuclei and affects the microphysics of cloud formation and precipitation over the rainforest.

Organic aerosols are ubiquitous in the atmosphere and play important roles in the climate system. They can cool Earth's surface by scattering sunlight or serve as nuclei for water droplets and ice crystals in clouds and precipitation. The properties and origin of organic aerosol particles are, however, still poorly understood, and their effects are among the largest uncertainties in the current understanding of climate (1–3). For reliable assessment and control of the human influence on climate, it is important to understand the natural background sources of atmospheric aerosols (4). One of the few continental regions where aerosols can be studied under near-natural conditions is the Amazon Basin, which has an aerosol burden that is mainly driven by an intensive biosphere-atmosphere interaction (5). Recent investigations indicate that the fine particles serving as cloud condensation nuclei (CCN) in pristine Amazonian rainforest air consist predominantly of secondary organic aerosol (SOA), formed by oxidation of

volatile organic compounds (VOC) and condensation of low- or semi-volatile oxidation products (6, 7). The actual mechanism of initial particle formation, however, remains unclear. In contrast to other vegetated continental regions, ultrafine particles with diameters < 30 nm (nucleation mode particles), which are characteristic for new particle-formation events in which gaseous species condense to form secondary aerosol particles, are almost never observed in pristine boundary layer air over the Amazonian rainforest (5, 8). One possible explanation for the lack of nucleation mode particles in the Amazonian boundary layer could be that the nucleation and the initial growth of new particles take place in the free troposphere, followed by downward transport in the course of convective overturning (9, 10). Alternatively, as we suggest here, the secondary organic material may condense onto preexisting primary particles directly emitted from the rainforest.

We applied scanning transmission x-ray microscopy with near-edge x-ray absorption fine

structure analysis (STXM-NEXAFS), scanning electron microscopy (SEM), and secondary ion mass spectrometry (NanoSIMS) to determine the microstructure and chemical composition of Amazonian organic aerosol particles in the accumulation mode (0.1 to 1 μm diameter). This size range is most relevant to the activation of cloud condensation nuclei (6). The aerosol samples were collected during the wet season (May 2011) at a remote rainforest site [Amazonian Tall Tower Observatory (ATTO) site] 150 km northeast of Manaus, Brazil. The investigated air masses came with the trade wind circulation from the northeast and traveled over some 1000 km of mostly pristine tropical rainforest. For comparison, we also investigated laboratory-generated SOA reference samples from isoprene and terpene oxidation, as well as reference samples generated by spray-drying of pure organic compounds in aqueous solution (11). We used STXM-NEXAFS for the determination of elemental and functional group composition in individual organic aerosol particles (12, 13) and SEM and NanoSIMS for further morphological characterization and independent confirmation of elemental composition.

The Amazonian aerosol samples comprised a mixture of homogeneous droplets and droplets containing internal structures that may be indicative of their atmospheric aging history (Fig. 1, A and B, and fig. S8). The NEXAFS spectra revealed characteristic similarities and differences between the chemical composition of the Amazonian aerosol and laboratory-generated reference samples. The terpene SOA reference particles exhibit a sharp peak representative of carboxylic acid groups (COOH), as well as shoulders indicating carbonyl groups (C=O) and carbon-carbon double bonds (C=C), but no pronounced signal of hydroxy groups (C-OH). Spectra of the isoprene SOA reference particles show a broad peak resulting from COOH and C-OH signals of comparable intensity, a C=O shoulder, and no C=C signal. Spectra of the carbohydrate reference particles exhibit a sharp C-OH peak, no COOH signal, and very weak C=O and C=C shoulders (Fig. 2A).

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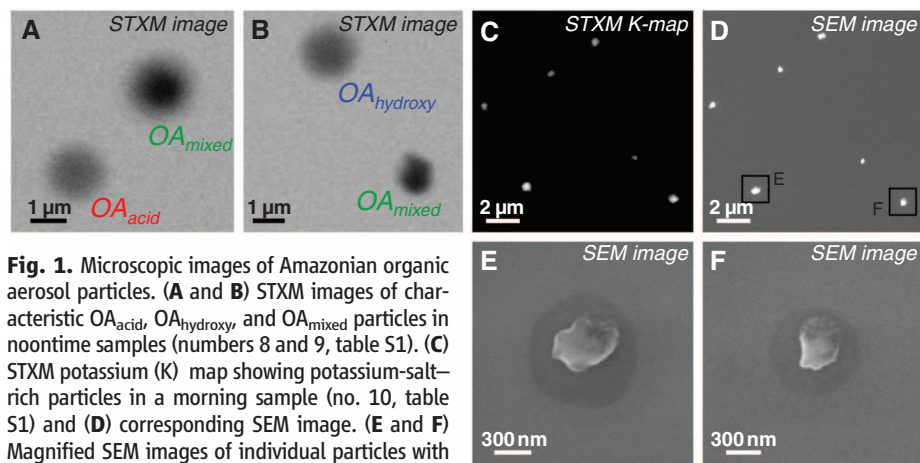


Fig. 1. Microscopic images of Amazonian organic aerosol particles. (A and B) STXM images of characteristic OA_{acid}, OA_{hydroxy}, and OA_{mixed} particles in noontime samples (numbers 8 and 9, table S1). (C) STXM potassium (K) map showing potassium-salt-rich particles in a morning sample (no. 10, table S1) and (D) corresponding SEM image. (E and F) Magnified SEM images of individual particles with salt core and organic coating [black frames in (D)].

In the Amazonian aerosol samples, three chemically distinct types of organic particles could be assigned to the following categories (Fig. 2A): (i) OA_{acid} particles exhibited spectra with a pronounced COOH peak similar to those of laboratory-generated SOA particles from terpene oxidation; (ii) $\text{OA}_{\text{hydroxy}}$ particles showed a strong hydroxy group signal similar to pure carbohydrate particles; and (iii) OA_{mixed} particles exhibited spectra resembling a mixture of OA_{acid} and $\text{OA}_{\text{hydroxy}}$ spectra. All three particle classes contained variable amounts of potassium. The coexistence of chemically distinct types of organic particles indicates the influence of different sources and formation mechanisms in the Amazonian boundary layer. Categories OA_{acid} and $\text{OA}_{\text{hydroxy}}$ each accounted for about 25% of all particles analyzed in this study, whereas OA_{mixed} was the most abundant particle type and contributed about 50%. Previous studies in Amazonia had shown that terpene- and isoprene-based SOA dominated the mass of organic aerosol (6, 7, 14), which is consistent with our observation of OA_{acid} , $\text{OA}_{\text{hydroxy}}$, and OA_{mixed} as a mixture of both. In addition to isoprene and terpene oxidation products, carbohydrates associated with primary particle emissions may also contribute to the observed organic matter (15, 16).

The most unexpected finding of our study was the presence of pronounced potassium signals in the NEXAFS spectra of nearly all analyzed organic particles (Fig. 2B). The potassium mass fraction is strongly size-dependent and decreases from ~20% at volume-equivalent particle diameters around 0.15 μm down to ~0.3% for diameters around 1 μm (Fig. 3), with a median value of 2.6% (11) (supplementary text S1.5). This observation suggests that small potassium-salt-rich particles from primary emissions act as seeds for the condensation of organic material and that the primary potassium content is diluted upon particle growth. The occurrence of these potassium-bearing particles has been confirmed by a combination of STXM, SEM, and NanoSIMS. In particular, samples collected during the morning hours show a high abundance of fine particles (~0.2 μm) with strong potassium signals and a low content of organic matter (Fig. 1, C to F, and fig. S7). The STXM and NanoSIMS results indicate that the potassium-rich particles also contain substantial quantities of ammonium cations as well as chloride and sulfate counteranions (11) (supplementary text S1.7).

Potassium-rich particles, in association with soot carbon, are an important component of biomass-burning smoke (17, 18). In our samples, however, we can exclude biomass burning as a source of the potassium-rich particles, because we did not find any particles containing soot carbon. Also, there were no fires detected in the region along the airmass trajectories during our study period (19, 20). Hence, biogenic emissions are the only potential source. Earlier investigations, including online high-resolution time-of-flight aerosol mass spectrometry (HR-ToF-AMS)

(fig. S12), had already reported substantial amounts of potassium associated with biogenic submicrometer aerosol in the Amazon during the wet season (21–23). They were not able to relate the presence of potassium to specific particle types, but the combination of potassium and sulfur has been attributed to local biogenic sources (24–26), which is consistent with the observation of potassium- and sulfate-rich particles in our study. The median atmospheric potassium concentration estimated from our analysis [$\sim 50 \text{ ng m}^{-3}$ for particles in the size range of 0.1 to 1 μm , (11) supplementary text S1.5] is consistent with previous measurement results [18 to 220 ng m^{-3} for particles $< 2 \mu\text{m}$ (16)]. Several studies show that active biota, such as plants and fungi, can efficiently release salts into the air (15, 16, 27–30). In particular, the active wet discharge of fungal spores is accompanied by the emission of aqueous droplets that contain potassium, chloride, and carbohydrates as the main osmolytes (11, 16) (supplementary text S2.1). STXM and light micrographs of our samples indicate a high abundance of fungal spores in the coarse particle fraction ($> 1 \mu\text{m}$, fig. S6), which supports the idea of fungal emissions as a plausible source for the observed potassium-rich particles.

SEM images show that the biogenic salt particles in the early morning samples consist of a

strongly electron-scattering salt core embedded in a thin organic coating (Fig. 1, E and F). However, potassium salt cores are not present in particles collected during the daytime. Instead, many particles show an inorganic microgranular material distributed over the entire particle (fig. S8, A and B). The phase separation observed in our samples follows the same pattern and dependence on oxygen-to-carbon ratio as reported in recent studies of liquid-liquid phase separation in organic and mixed organic-inorganic aerosol particles (31–33): $\text{OA}_{\text{hydroxy}}$ particles with high atomic ratios of oxygen to carbon ($\text{O:C} \approx 0.9$ to 1.0) showed no phase separation, whereas OA_{acid} and OA_{mixed} particles with O:C ratios around 0.5 to 0.7 showed internal structures with a COOH-rich core and a C–OH-rich shell (table S4 and fig. S8). These observations indicate a pronounced influence of aqueous processing in deliquesced aerosol particles and cloud or fog droplets on the growth and aging of SOA particles, that is, the formation and evaporation of aqueous droplets in which multiphase chemical reactions can produce secondary organic matter and the inorganic salt seeds can undergo cyclic dissolution and recrystallization. SOA formation by multiphase rather than gas-phase chemistry might also contribute to a suppression of new particle formation (11) (supplementary text S2.3 and S2.4).

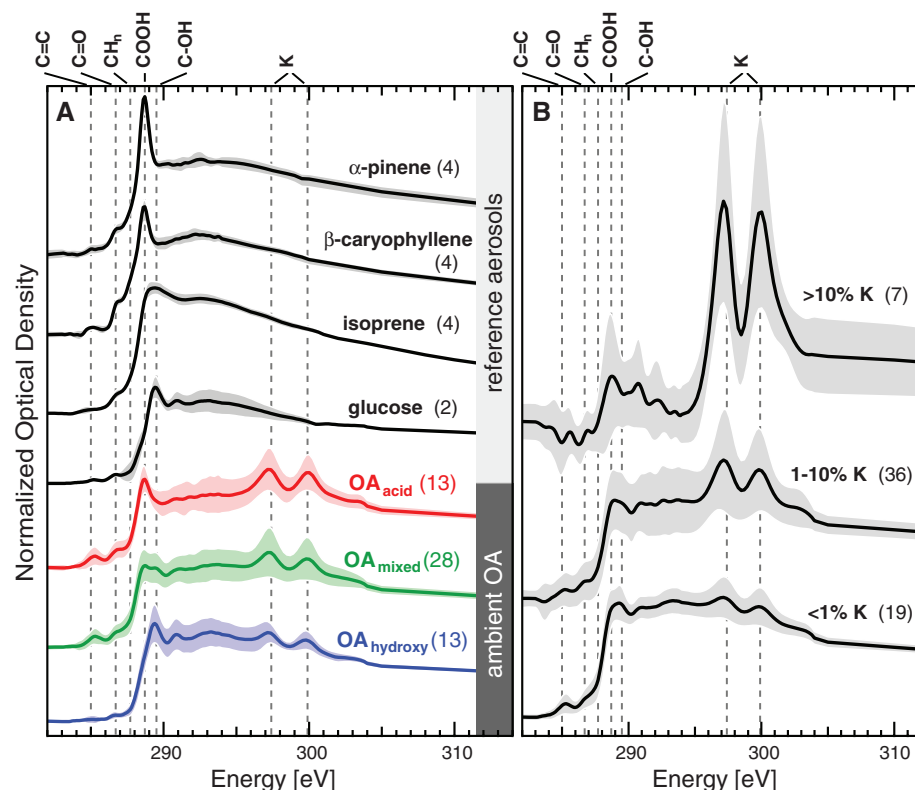


Fig. 2. (A) NEXAFS spectra of (i) laboratory-generated SOA from terpene and isoprene oxidation; (ii) glucose as carbohydrate reference compound from spray-drying of aqueous solution; and (iii) OA_{acid} , OA_{mixed} , and $\text{OA}_{\text{hydroxy}}$ particles from the Amazon. (B) NEXAFS spectra for Amazonian organic aerosol particles with different potassium (K) mass fractions. Solid lines and shaded areas represent mean spectra and standard deviations. Numbers of analyzed particles are given in parentheses. Vertical lines indicate resonant absorption of organic functional groups and potassium (table S3).

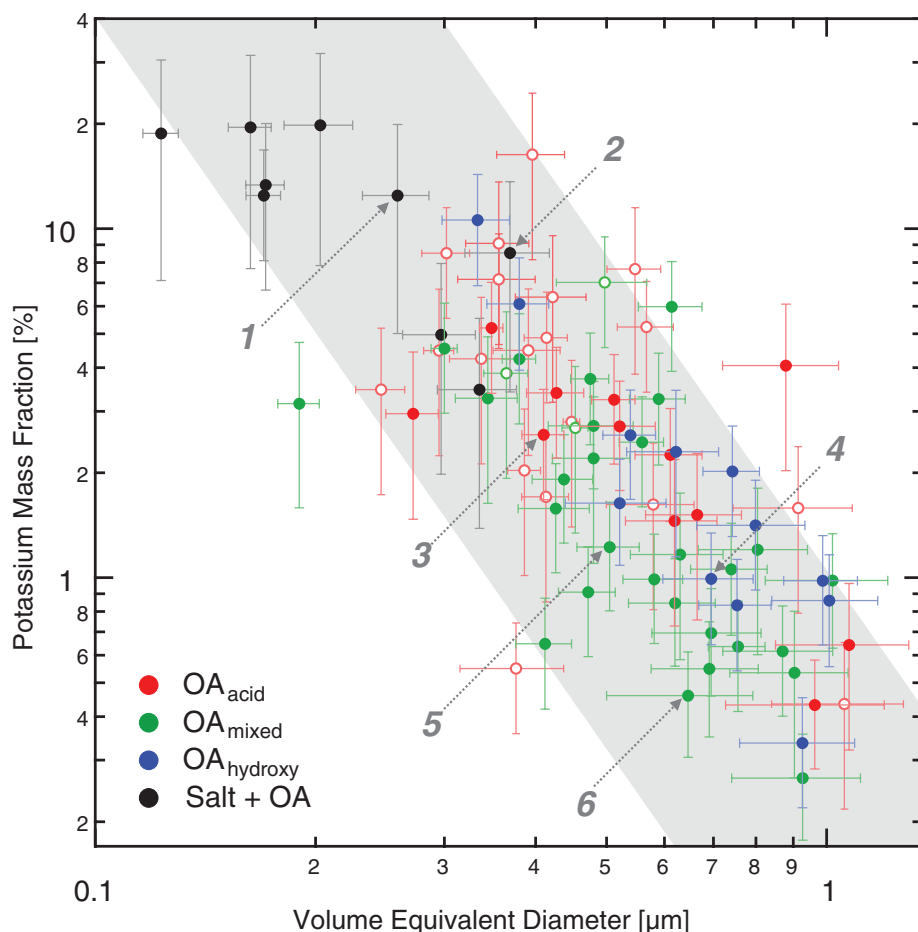
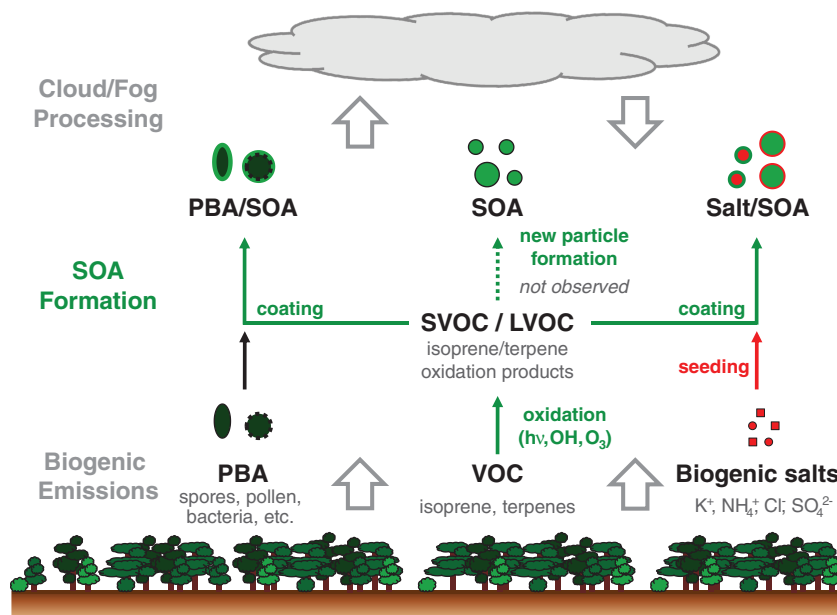


Fig. 3. Size dependence of potassium mass fraction in Amazonian organic aerosol particles. Solid markers represent data from samples collected in this study (ATTO site, 2011), and open markers represent additional data from previously collected samples (ZF2 site, 2010) (11). Numbers and arrows identify individual particles from Fig. 1 (1, F; 2, E; 3 and 5, A; and 4 and 6, B). Shaded area illustrates idealized dilution of primary potassium content upon particle growth by condensation of secondary organic material (inverse third-order dependence on particle diameter) (11). Error bars indicate the estimated uncertainty in calculations of particle size and potassium mass content.

Fig. 4. Sources and processing of organic aerosol in pristine Amazonian boundary layer air. SOA formation by photooxidation of VOC and condensation of semi- and low-volatile organic compounds (SVOC/LVOC) on primary biological aerosols (PBA) that dominate the coarse particle fraction ($>1\ \mu\text{m}$) (5, 6) and on biogenic salt particles that serve as seeds for organic particles dominating the accumulation size range (0.1 to $1\ \mu\text{m}$).



Of the 77 Amazonian organic aerosol particles analyzed by STXM-NEXAFS, only 3 contained no detectable amount of potassium ($<2\ \text{fg}$). The near-ubiquitous presence of potassium suggests that biogenic salt particles emitted from active biota in the rainforest serve as initial seeds for the condensation of VOC oxidation products. This mechanism appears to dominate the formation of SOA particles in the accumulation size range in pristine Amazonian rainforest air (Fig. 4). It can explain why new particle formation events are not observed, even though the aerosol consists largely of secondary organic material formed from gas-phase precursors (11) (supplementary text S2.4). A major implication is that the number concentration of atmospheric aerosol particles in the accumulation size range is partly regulated by the primary emission of potassium-salt-rich particles from biota in the rainforest. Compared with smaller particles in the nucleation and Aitken size range ($<0.1\ \mu\text{m}$), accumulation mode particles are by orders of magnitude more frequently activated as CCN (11) (supplementary text S2.3 and fig. S11B). Thus, the biological sources and emission rates of potassium-salt-rich particles have a direct influence on the initial droplet number and microphysical evolution of clouds over the rainforest, which in turn influence the dynamics of clouds and precipitation as well as their effects on the hydrological cycle and climate.

Our findings support the hypothesis that the Amazonian rainforest ecosystem can be regarded as a biogeochemical reactor in which the formation of clouds and precipitation in the atmosphere are triggered by particles emitted from the biosphere. The connection between biogenic particle emissions and cloud properties in the tropical rainforest ecosystem appears even stronger and more direct than previously assumed (6, 34). In view of the large impact of tropical rainforests on biogeochemistry and climate, the biological

activity and diversity of particle-emitting organisms seem likely to play important roles in Earth history and future global change.

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Acknowledgments: This work has been supported by the Max Planck Society, the Max Planck Graduate Center, the Geocycles Cluster Mainz (Landesexzellenzcluster Rheinland-Pfalz),

and the European Community (PEGASOS, FP7-265148). The Harvard Environmental Chamber was supported by the Office of Science, Office of Basic Energy Sciences (BES), U.S. Department of Energy (DOE), grant no. DE-FG02-08ER6452, and the U.S. NSF under grant no. 0925467. The Advanced Light Source is supported by the Director, Office of Science, BES, of the U.S. DOE under contract no. DE-AC02-05CH11231. We thank the Helmholtz-Zentrum Berlin for the allocation of synchrotron radiation beamtime at BESSY II. We thank the Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus, and the ATTO team under the Brazilian coordinator, A. O. Manzi, for their collaboration and field support. We also thank G. R. Carmichael and the Center for Global and Regional Environmental Research at the University of Iowa for support in the Weather Research and Forecasting (WRF) model simulations. We gratefully acknowledge R. Ditz, I. Trebs, X. Chi, J. A. Huffman, J. Kesselmeier, J. Schöngart, M. Kuwata, T. Tyliczszak, J. Huth, G. Schütz, E. Goering, M. Bechtel, J.-D. Förster, and T. Behrendt for support and helpful discussions.

Supplementary Materials

www.sciencemag.org/cgi/content/full/337/6098/1075/DC1
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12 April 2012; accepted 23 July 2012
10.1126/science.1223264

Radiative Absorption Enhancements Due to the Mixing State of Atmospheric Black Carbon

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Atmospheric black carbon (BC) warms Earth's climate, and its reduction has been targeted for near-term climate change mitigation. Models that include forcing by BC assume internal mixing with non-BC aerosol components that enhance BC absorption, often by a factor of ~2; such model estimates have yet to be clearly validated through atmospheric observations. Here, direct in situ measurements of BC absorption enhancements (E_{abs}) and mixing state are reported for two California regions. The observed E_{abs} is small—6% on average at 532 nm—and increases weakly with photochemical aging. The E_{abs} is less than predicted from observationally constrained theoretical calculations, suggesting that many climate models may overestimate warming by BC. These ambient observations stand in contrast to laboratory measurements that show substantial E_{abs} for BC are possible.

Black carbon (BC) in the atmosphere has a strong effect on global and regional climate, with some estimates suggesting that the positive (warming) radiative forcing by BC is second only to CO₂ (1), making it an important near-term climate mitigation target (2, 3). Quantification of the warming caused by BC in global climate models depends explicitly on the mixing state assumed for particles (internal versus external) and, for internal mixtures, the assumed influence of coatings on the magnitude of BC absorption (4–6). Optical properties of internally mixed BC-containing particles can be calculated in various ways, all of which indicate substantially greater absorption than for an equivalent exter-

nal mixture—the absorption by internally mixed BC is “enhanced” because the coatings act as a lens (7). Model estimates of BC radiative forcing are increased by up to a factor of 2 for internally versus externally mixed BC (4, 5), and many models that use external mixtures simply multiply BC absorption by a scaling factor (8) to account for the theoretical absorption enhancement (E_{abs}). However, the magnitude of E_{abs} has not been determined for real atmospheric particles (9, 10), which is crucial as more models describe aerosol distributions as combinations of internal and external mixtures (11).

In this study, direct measurements of E_{abs} and average mixing state for BC in the atmo-

sphere around California are reported from two field campaigns: the 2010 CalNex study and the Carbonaceous Aerosols and Radiative Effects Study (CARES). The CalNex measurements were made onboard the R/V *Atlantis*, whereas the CARES measurements were made at a ground site in the Sacramento urban area (fig. S1) (12). Our observations indicate that the E_{abs} for ambient particles around large urban centers do not vary much with photochemical aging, are significantly less than predicted from traditional core-shell Mie theory, and are in contrast to laboratory experiments, suggesting that the warming by BC may be overestimated in climate models. Further, they indicate a role for absorption by non-BC aerosol components [brown carbon (BrC)] (13) in urban environments at short visible wavelengths.

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Photochemical aging of urban air masses leads to the production of nonrefractory particulate matter (NR-PM), some of which is internally mixed with BC and can, in principle, lead to $E_{\text{abs}} > 1$. The fraction of NR-PM exclusively associated with BC is termed here NR-PM_{BC}. The extent to which BC can theoretically be enhanced via lensing depends critically on the ratio $R_{\text{BC}} = [\text{NR-PM}_{\text{BC}}]/[\text{BC}]$ (7). During CalNex, chemically resolved mass concentrations of sub-micrometer NR-PM_{BC} were explicitly measured with a SP-AMS (soot particle–aerosol mass spectrometer) (14), from which R_{BC} is directly quantified. The observed R_{BC} increases rapidly with photochemical age (PCA), which was estimated from the quantity $-\log([\text{NO}_x]/[\text{NO}_y])$ (Fig. 1A). [The ratio $-\log([\text{NO}_x]/[\text{NO}_y])$ serves as a photochemical “clock” by assuming that the conversion of NO_x ($=\text{NO} + \text{NO}_2$) to NO_y occurs at a rate equal to the $\text{NO}_2 + \text{OH}$ reaction rate (12).] This indicates that photochemical aging led to significant pro-

duction of NR-PM_{BC} material and growth of BC-containing particles, in particular through condensation of oxygenated organic aerosol (OOA) and SO_4^{2-} (Fig. 1, B to D, and figs. S8 and S9). These measurements show explicitly how the composition of only the BC-containing particles changes during photochemical aging, providing strong constraints for use in comparing the observed E_{abs} with theoretical calculations.

During CalNex and CARES, E_{abs} was measured as the ratio between ambient particle absorption ($b_{\text{abs,ambient}}$) and the absorption after particle heating in a thermodenuder ($b_{\text{abs,TD}}$) to evaporate and remove non-BC NR-PM, including NR-PM_{BC} (fig. S4) (12). The absorption measurements were made at 532 and 405 nm by using photoacoustic spectroscopy (fig. S3) (12). The observed E_{abs} include effects of both lensing and of BrC absorption (15).

Despite the substantial photochemical production of NR-PM and NR-PM_{BC} and the growth

of BC-containing particles (Fig. 1), the observed E_{abs} values during both campaigns change only slowly with PCA and are not much above unity (Fig. 2). Further, the E_{abs} during CalNex exhibited minimal dependence on R_{BC} (Fig. 3). The average $E_{\text{abs,532nm}}$ during both campaigns is 1.06 ± 0.006 (2 SEM), suggesting that NR-PM_{BC} increased the absorption by 6% on average. The slightly larger E_{abs} at 405 nm [1.13 ± 0.01 (2 SEM)] likely indicates the influence of BrC on absorption in this wavelength region. Consideration of the BC mass absorption coefficient ($\text{MAC}_{\text{BC}} = b_{\text{abs}}/[\text{BC}]$), variations in which have traditionally been used to infer E_{abs} , leads to similar conclusions (although with greater uncertainties) (fig. S17) (12). Overall, these results lead to the unexpected conclusion that photochemical aging and NR-PM_{BC} production did not cause a substantial increase in the absorption enhancement for BC. Single-particle microscopy measurements from locations around the world (16–18) indicate it is common to find BC inclusions at the edge of collected particles rather than deeply embedded in a “coating” material (which would be necessary to observe large absorption enhancements), which is consistent with our ambient observations.

Climate models that account for internal mixing of BC commonly use core-shell Mie theory to calculate the optical properties of BC-containing particles. Time-series of E_{abs} during CalNex have therefore been calculated here by using core-shell Mie theory and binned according to PCA. One feature of our study is that all inputs to the calculations, in particular the particle mixing state (the R_{BC} and size distributions of both BC and non-BC containing particles), were observationally constrained by the comprehensive suite of instrumentation available during CalNex (12). E_{abs} was calculated for either a unimodal or bimodal distribution of coating thicknesses on the BC particles (fig. S7) (12). The calculated E_{abs} are significantly greater than the observed values at all PCAs, demonstrating that core-shell Mie theory substantially overestimates the actual E_{abs} , even when explicitly constrained by observations of R_{BC} (Fig. 2). The difference between the bimodal and unimodal simulations illustrates the importance of mixing state assumptions to the calculations. If all NR-PM (not just NR-PM_{BC}) had been assumed to be internally mixed with BC, the overprediction of E_{abs} would have been even larger because only ~20% of the total sub-micrometer NR-PM was NR-PM_{BC}, on average. This is an important consideration for models that assume internal mixing but do not dynamically account for the distribution of NR-PM between BC and non-BC-containing particles.

These ambient observations are in contrast to results from laboratory experiments we conducted in which large $E_{\text{abs,532nm}}$ values were observed when flame-generated BC was internally mixed with dioctyl sebacate (DOS) (Fig. 3) (12). For a given BC particle size, the measured E_{abs} increased with R_{BC} (which varied over the same range as the ambient R_{BC}) and were generally consistent with

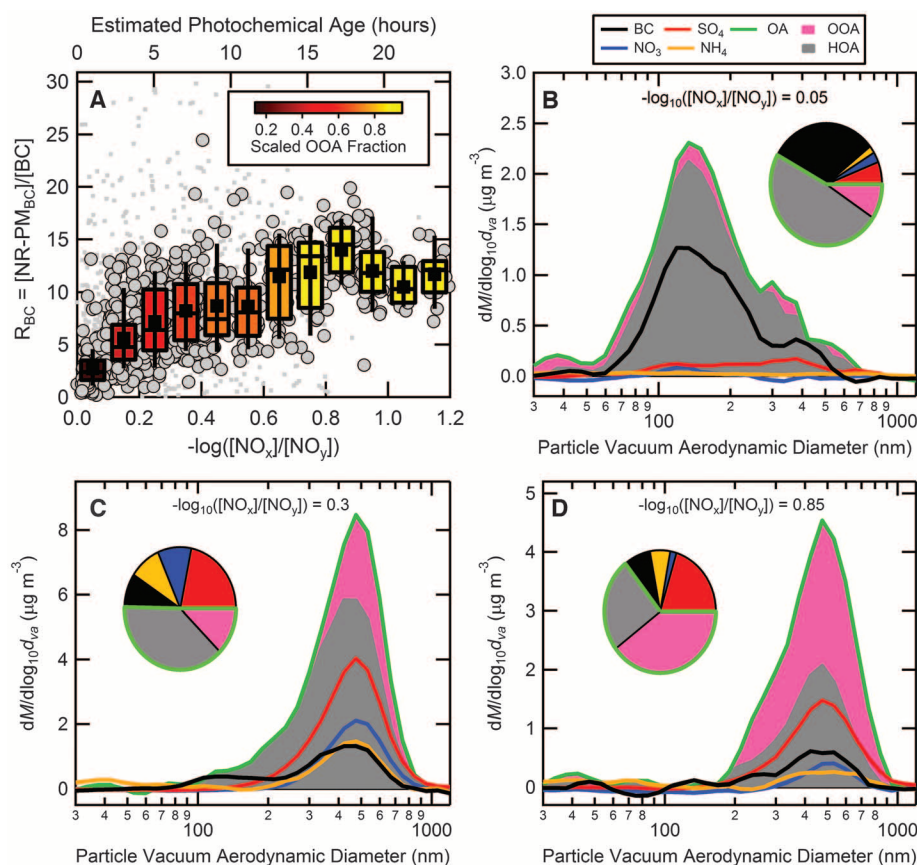


Fig. 1. (A) The R_{BC} ($=[\text{NR-PM}_{\text{BC}}]/[\text{BC}]$) ratio as a function of PCA ($-\log([\text{NO}_x]/[\text{NO}_y])$) for total NR-PM_{BC} during CalNex. The box and whisker plots show the mean (■), median (—), lower and upper quartile (boxes), and 9th and 91st percentile (whisker) results for periods in which $[\text{BC}] > 0.07 \mu\text{g m}^{-3}$ (light gray points, ●). For reference, the gray dots show all data. The corresponding PCA (assuming $[\text{OH}] = 4 \times 10^6 \text{ molecules cm}^{-3}$) is shown on the top axis. The boxes are color-coded according to the scaled oxygenated organic aerosol (OOA) fraction of total OA (12). (B to D) Chemically resolved mass-weighted particle time-of-flight vacuum aerodynamic diameter (d_{va}) size distributions from the SP-AMS for BC internally mixed with NR-PM_{BC}, including SO_4^{2-} , NO_3^- , NH_4^+ , and OA, for periods where $-\log([\text{NO}_x]/[\text{NO}_y])$ was (B) 0.05 (fresh; $R_{\text{BC}} = 3.1$), (C) 0.3 (intermediate; $R_{\text{BC}} = 10.3$), and (D) 0.85 (aged; $R_{\text{BC}} = 15.8$). The total OA has been split into two OA types identified from factor analysis as hydrocarbon-like organic aerosol (HOA) and OOA. The pie charts show the fractional contributions of the various species to the total mass of BC-containing particles.

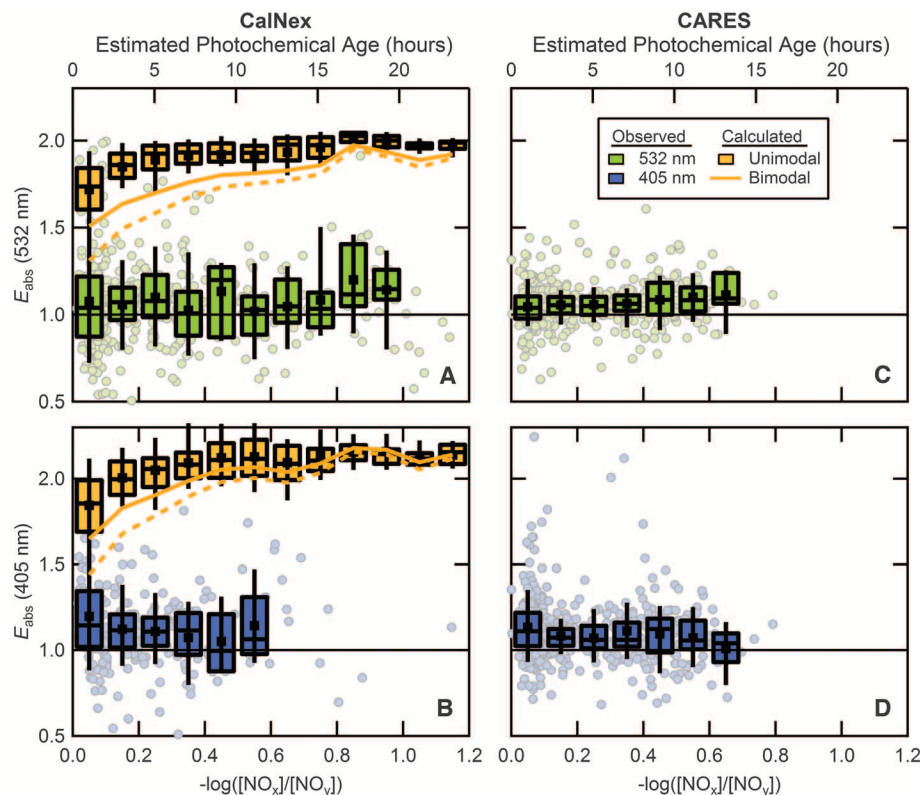
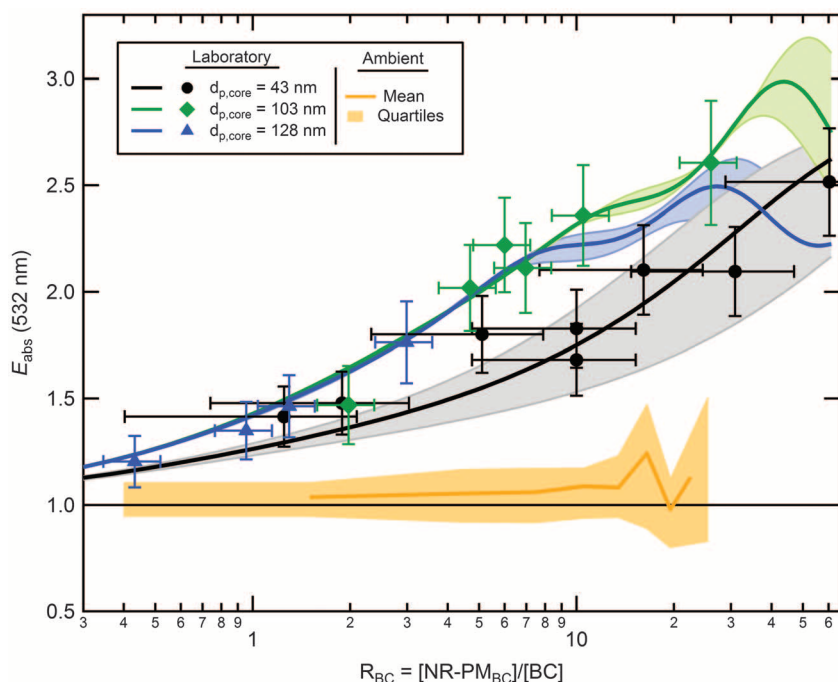


Fig. 2. Measured E_{abs} at (A and C) 532 nm (green) and (B and D) 405 nm (blue) for CalNex [(A) and (B)] and CARES [(C) and (D)] as a function of PCA, estimated from $-\log([NO_x]/[NO_y])$. The light-colored points correspond to individual measurements, whereas the box and whisker plots show the binned mean ($\blacksquare$), median (—), lower and upper quartile (boxes), and 9th and 91st percentile (whisker). Calculated E_{abs} values for CalNex are shown, assuming that the distribution of NR-PM_{BC} material on the BC-containing particles was either bimodal (orange lines) or unimodal (orange box and whisker). For the bimodal case, one mode was assumed to be “thick” coated, whereas the other was “thin” coated. The thinly coated mode was assumed to have either $R_{\text{BC}} = 1$ (solid) or 0.1 (dashed).

Fig. 3. Observed E_{abs} at 532 nm as a function of R_{BC} for laboratory experiments in which BC particles of various size, produced from ethylene flame, were coated with dioctyl sebacate (symbols). The $d_{\text{p,core}}$ values are the volume-equivalent diameter of the uncoated BC particles. Uncertainties are 1σ . Calculated E_{abs} from core-shell Mie theory (lines) are shown for the differently sized BC particles and are in generally good agreement with the observations; the colored bands show the uncertainty range in the calculations. The observed mean ambient particle E_{abs} versus R_{BC} during CalNex is shown for comparison (orange line).



core-shell theory. These laboratory results clearly demonstrate that internal mixing of BC with NR-PM can produce large E_{abs} , as has previously been observed (19, 20). Further work is needed to resolve the discrepancies between field observations and laboratory studies.

Although there is no evidence of strong lensing-induced absorption enhancements at 532 nm, the slightly larger E_{abs} at 405 nm suggests absorption by some NR-PM species at shorter wavelengths occurred; we assume the absorbing NR-PM species to be BrC. BrC is particulate organic carbon that absorbs light at visible and near-ultraviolet (UV) wavelengths (13), with the absorption increasing strongly toward shorter wavelengths (21). Here, the difference between $E_{\text{abs},405\text{nm}}$ and $E_{\text{abs},532\text{nm}}$ can be interpreted as the approximate contribution to absorption by BrC at 405 nm—whether it exists internally mixed with BC or not (15). For both CalNex and CARES, BrC absorption is $\sim 10\%$ of the total absorption at 405 nm (Fig. 2), corresponding to campaign average MACs for BrC of $0.12 \text{ m}^2/\text{g}$ (CalNex) and $0.14 \text{ m}^2/\text{g}$ (CARES) and a derived imaginary refractive index of ~ 0.004 (12). This additional absorption by NR-PM in the near-UV region can have an impact on photochemical O_3 production (22, 23) and could suppress OH concentrations, thus increasing the lifetime of greenhouse gases such as methane or affecting the conversion of SO_2 into scattering sulfate aerosol (24).

Our measurements indicate that BC emitted from large to medium-sized urban centers (dominated by fossil fuel emissions) does not exhibit a substantial absorption enhancement when internally mixed with non-BC material, which is in stark contrast to laboratory experiments and model calculations. The small observed values

of E_{abs} suggest that models that assume internal mixtures in a core-shell configuration, or scale the absorption (or forcing) by externally mixed BC particles, can substantially overestimate the atmospheric warming by BC, potentially by up to a factor of 2 (4, 5). The climate benefits of BC mitigation (3) would similarly be overestimated. This would be true even for models that specifically track the mixing state of BC particles as they evolve in time (25). It is possible that non-fossil-derived BC (such as emitted from biomass burning) may exist with a considerably different internal morphology or amounts of BrC as compared with the ambient particles observed in this study, and thus different observable E_{abs} values. Models may ultimately need to treat BC from fossil-fuel combustion differently than BC from biomass burning, although this awaits validation through further measurements of wavelength-dependent E_{abs} for atmospheric particles in a variety of locations around the world. The contrast between our ambient observations and model formulations highlights the still incomplete understanding of radiative forcing by atmospheric BC with respect to particle-mixing state. Additional challenges include the quantification of BC emission inventories, wet-deposition removal rates,

and the specification of the spatial and temporal distributions of BC (particularly the altitudinal profile) (26).

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Acknowledgments: The authors thank the crew of the R/V *Atlantis*, the support at American River College and Cambustion for loan of the centrifugal particle mass analyzer (CPMA). This work was supported by the NOAA Climate Program Office (NA09OAR4310124 and NA09OAR4310125), U.S. Environmental Protection Agency (RD834558), NSF Atmospheric Chemistry, California Air Resources Board, U.S. Department of Energy (DOE) Atmospheric System Research (ASR) program and DOE Atmospheric Radiation Measurement (ARM) Climate Research Facility, the Canadian Federal Government (PERD Project C12.007), and the Natural Sciences and Engineering Research Council of Canada.

Supplementary Materials

www.sciencemag.org/cgi/content/full/337/6098/1078/DC1

Materials and Methods

Figs. S1 to S19

References

17 April 2012; accepted 3 July 2012

10.1126/science.1223447

A Gain-of-Function Polymorphism Controlling Complex Traits and Fitness in Nature

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Identification of the causal genes that control complex trait variation remains challenging, limiting our appreciation of the evolutionary processes that influence polymorphisms in nature. We cloned a quantitative trait locus that controls plant defensive chemistry, damage by insect herbivores, survival, and reproduction in the natural environments where this polymorphism evolved. These ecological effects are driven by duplications in the *BCMA* (branched-chain methionine allocation) loci controlling this variation and by two selectively favored amino acid changes in the glucosinolate-biosynthetic cytochrome P450 proteins that they encode. These changes cause a gain of novel enzyme function, modulated by allelic differences in catalytic rate and gene copy number. Ecological interactions in diverse environments likely contribute to the widespread polymorphism of this biochemical function.

Few studies have identified the genes that underlie complex trait variation in nature and the evolutionary processes that influence these polymorphisms. Most such work has focused on loss-of-function mutations that lead to adaptive phenotypes (1), likely because novel gain-of-function changes occur infrequently and require persistent natural selection to be maintained in populations (2). Nonetheless, new functional mechanisms are crucially important for adaptive evolution (3). To understand the adaptive consequences of complex trait variation, we

must establish a direct relationship between genetic polymorphisms and phenotypic traits, and investigate the fitness consequences of this variation in natural environments (1).

Glucosinolates are biologically active secondary compounds (fig. S1) found in *Arabidopsis* and its relatives (4) that are important in many aspects of plant defense, influencing oviposition and feeding by insect herbivores (5), defense against microbial pathogens (6), and composition of associated microbial communities (7). Typically, generalist insects are sensitive to glucosinolate-

based plant defenses, whereas specialists may be able to cope with these compounds, which may serve as oviposition cues and feeding stimulants (5).

The ecological model plant *Boechera stricta* (Brassicaceae) is a native, short-lived perennial with a close phylogenetic relationship to *Arabidopsis* (8), often found in undisturbed habitats where current environments are similar to historical conditions that have existed for ~3000 years (9). In field populations near Lost Trail Pass in Montana and Crested Butte in Colorado, we measured natural selection on foliar damage from herbivores using local genotypes. We mapped a quantitative trait locus (QTL) in *B. stricta* that contributes to insect resistance and controls allocation to glucosinolates derived from branched-chain amino acids or methionine [the *BCMA* (branched-chain methionine allocation) locus] (10). Although most Brassicaceae synthesize glucosinolates from

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methionine or tryptophan precursors, among the genera closely related to *Arabidopsis*, only *Boechea* produces glucosinolates from branched-chain amino acids, specifically valine or isoleucine (11). We refer to these two categories of methionine-derived and branched-chain glucosinolates as Met-GS and BC-GS, respectively.

We examined variation in *B. stricta* for three herbivory-related traits: leaf damage by herbivores, total glucosinolates, and BC ratio (i.e., the proportion of aliphatic glucosinolates derived from valine or isoleucine) in nine natural populations in Idaho and Montana, and found significant genetic variation for all traits (table S1) (12). Levels of herbivore damage (percentage of leaf area removed) and total quantity of foliar glucosinolates showed genetic variation (Fig. 1 and table S1; $P = 0.0355$ for herbivore damage among families, other $P < 0.0001$) and continuous phenotypic distributions typical of complex traits. In contrast, we found a discontinuous distribution in the proportion of aliphatic BC-GS versus Met-GS (Fig. 1), which corresponds to the *BCMA* QTL (12, 13). The parental genotypes examined here have glucosinolate phenotypes that are representative of other plants in these populations (12).

We quantified the ecological effects of this variation by measuring late-season foliar glucosinolates in 1030 F_6 near-isogenic line (NIL) plants in the Montana (MT) and Colorado (CO) field sites. Segregation of the *BCMA* locus predicted the BC ratio in both environments ($P < 10^{-199}$;

table S2) and both were significant predictors of insect damage ($P < 0.008$; table S3). Early-season herbivory showed significant effects of *BCMA* in Montana ($P < 10^{-6}$; table S4) but not in Colorado. However, the *BCMA* $\times$ site interaction for herbivore damage was not significant early in the season ($P = 0.092$; table S4) or for maximum damage on plants that survived through the summer (table S3). The quantitative level of leaf damage was a significant predictor of mortality in both Montana and Colorado ($P = 0.0017$; table S5), with no hint of heterogeneous selection gradients among sites (damage $\times$ site interaction, $P = 0.48$). Combining the observed levels of damage and estimates of natural selection (Fig. 2 and table S4), we calculate that the *BCMA-MT* homozygote had 1.3% higher fitness than the *BCMA-CO* genotype (12).

We compared herbivore damage and fecundity on 1435 recombinant inbred line (RIL) plants, in 2009 in Montana and in 2010 in Colorado. Mean herbivore damage was higher in Colorado ($64.2 \pm 5.8\%$) than in Montana ($10.3 \pm 1.9\%$). *BCMA* genotype predicted herbivore damage in Montana, with the *BCMA-CO* homozygote showing higher damage than the native *BCMA-MT* genotype (14.0 and 9.0%, respectively, $P < 0.0001$; table S6). In contrast, *BCMA* genotypes showed no significant difference in damage levels in Colorado ($P = 0.63$), perhaps because herbivores in Colorado are resistant to these compounds, or because chemical defenses were overwhelmed by high

levels of herbivory. This *BCMA* $\times$ site interaction for herbivore damage was significant ($P = 0.019$; table S6). In addition, herbivore damage was a significant predictor of fecundity in Montana (Fig. 2, $P < 0.013$; table S7) but not in Colorado ($P = 0.72$), with a significant *BCMA* $\times$ site interaction for probability of fruiting ($P = 0.05$; table S8). In Montana the local allele enhances the probability of fruiting by 132% relative to the Colorado allele (12); however, there is no effect of allelic variation in Colorado. Combining observed levels of damage and estimates of natural selection, we calculate that the *BCMA-MT* homozygote had 12% higher fecundity in Montana than did the Colorado homozygote (Fig. 2 and table S6) (12). Such large fitness differences may explain why many populations are nearly fixed for *BCMA* (Fig. 1B). Overall, the protective effect of *BCMA* against herbivory appears to differ between sites (table S6), whereas substantial fitness reduction due to leaf damage is commonly observed across sites and years (Fig. 2).

To control for the effects on fitness caused by linked genes, as well as other selective factors that might be correlated with herbivore damage, we planted 1539 F_6 NIL plants where each was assigned to an undamaged control group or to artificial herbivory (removal of $\sim 33\%$ of each leaf) (12). On average, a loss of 1% of leaf area caused a 1.2% reduction in survival (Fig. 2), with significantly elevated mortality in the herbivory treatment ($P < 0.0001$; table S9).

In *Arabidopsis thaliana*, the *CYP79F* locus encodes the first step of the core Met-GS pathway (14, 15) and *CYP83A* encodes the second step (16, 17). We sequenced bacterial artificial chromosomes (BACs) from both genotypes that gave rise to the RIL and NIL populations, and identified nine markers within the 1-cM interval

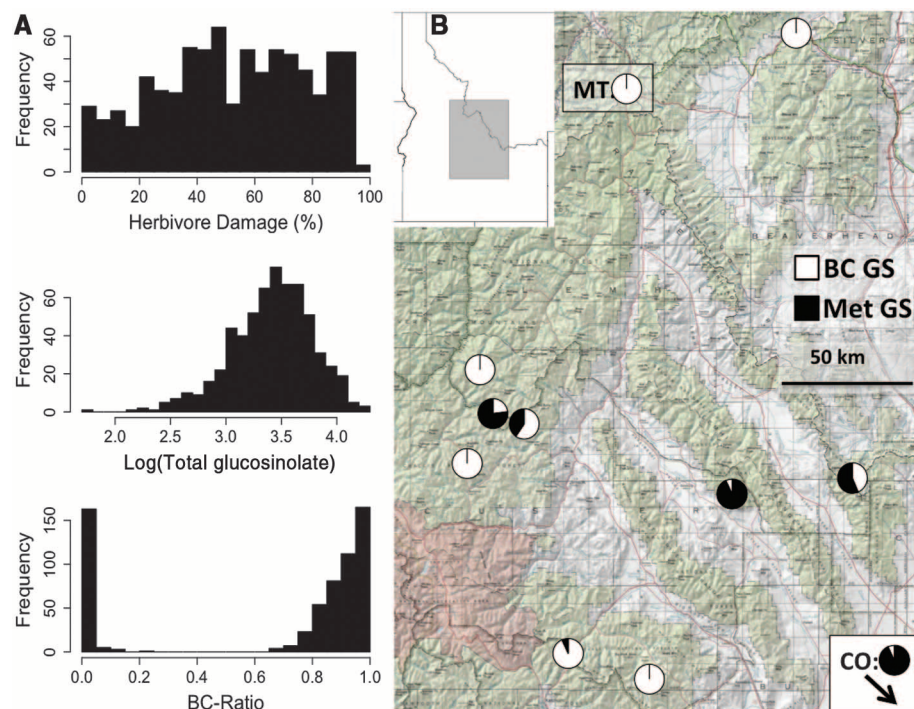


Fig. 1. (A) Histograms show percent leaf area removed by the generalist herbivore *Trichoplusia ni*, total quantity of glucosinolates, and proportion of aliphatic glucosinolates from branched-chain amino acid precursors (BC-GS). Greenhouse-grown plants were descended from nine *B. stricta* populations. (B) Map showing the proportion of genotypes in each population that produce predominantly BC-GS (white) or Met-GS (black). Parental populations of the crossing experiment are boxed.

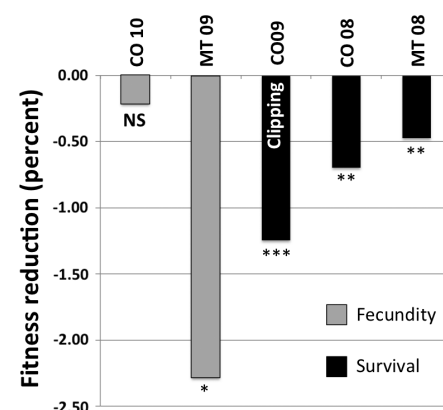


Fig. 2. Fitness reductions under field conditions associated with 1% loss of leaf area by herbivory. Bars indicate reduction in components of fitness due to fecundity (gray) and survival (black) in 2008, 2009, and 2010 in Colorado and Montana. NIL plants in the clipping experiment were randomly assigned to artificial herbivory or control treatments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; NS, not significant.

containing the *BCMA* QTL. These included sequences orthologous to *CYP79F* and *CYP83A*. *CYP83A* is 0.33 cM from the peak lod score (logarithm of the odds ratio for linkage) for the *BCMA* QTL (fig. S2 and table S10), whereas the *CYP79* polymorphism has a peak lod score of 365.3, with 10-lod confidence interval <0.1 cM wide (fig. S2 and table S10).

We created transgenic *Arabidopsis* plants for each locus and allele of the *CYP79* gene family from *Boechera* (12). Phenotypes of the transgenic plants show that the *BCMA* biochemical polymorphism is controlled by these *CYP79* loci, hence *BCMA* is a gene family with three expressed copies

(fig. S3). These enzymes convert amino acids to their corresponding oximes in the first step of glucosinolate biosynthesis (15). *BCMA2* is syntenic with the *CYP79F1* region in *A. thaliana* but is not linked to the *BCMA* QTL that controls the BC ratio. *BCMA3* and *BCMA1* are tightly linked at the LOD peak of the *BCMA* QTL, with *BCMA3* present in both parental genotypes, whereas *BCMA1* is only present in the Montana genotype.

We expressed these *BCMA* sequences and controls in 130 independent *Arabidopsis* transformants to control for position effects and number of insertions. We found significant differences in foliar glucosinolates derived from methionine,

valine, or isoleucine in transgenic plants ($P < 10^{-45}$; Fig. 3A, fig. S4, and table S11). *BCMA1-MT* transgenics showed increased production of isoleucine-derived glucosinolates ($P < 0.04$), and both *BCMA3* alleles caused increased production of valine-derived glucosinolates ($P = 0.034$ and $P = 0.0001$ for *BCMA3-CO* and *BCMA3-MT*, respectively) relative to controls. In addition, *BCMA1-MT* and both *BCMA2* alleles caused modest increases in methionine-derived glucosinolates ($P = 0.028$ to $P = 0.002$).

We compared *BCMA3-MT* and *BCMA3-CO* transgenics and found no significant difference for total concentration of aliphatic glucosinolates ($P > 0.05$; table S11). However, the *BCMA3-MT* allele produced higher levels of valine glucosinolates than did the *BCMA3-CO* allele (factor of 3.5; $P = 0.0002$). The *CO* allele differs from *BCMA3-MT* by an amino acid substitution in the substrate-binding region, which may be responsible for this reduced concentration of valine glucosinolates in transgenic plants. Finally, allele-specific expression to test for cis-regulatory variation found no significant differences in gene expression (12).

Heterologous expression in *Escherichia coli* also indicated that the enzymes encoded by *BCMA1* and *BCMA3*, but not *BCMA2*, have acquired catalytic activity toward branched-chain amino acid precursors (the “BC-AA clade,” which includes an orthologous sequence from *B. retrofracta*, which also produces BC-GS) (11). Comparing the rate of nonsynonymous versus synonymous substitution with maximum likelihood in PAML (18) indicated that the branch leading to the BC-AA clade (branch F in fig. S5) has undergone accelerated biochemical evolution ($P = 0.036$; table S12). In addition, two amino acid sites (134 and 536) in the BC-AA clade also showed rapid evolution (table S13).

The *BCMA1-MT* enzyme has evolved elevated activity toward isoleucine (Fig. 3B and table S14). For valine, we found significant catalytic activity for *BCMA1* and *BCMA3* and a modest increase for *BCMA2*. Although transgenic analysis showed that the *BCMA3-MT* allele produced higher levels of valine glucosinolates than the *BCMA3-CO* allele, heterologous expression did not detect a significant difference in the rate of valine catalysis between these *BCMA3* alleles ($t = 1.09$, $df = 6$, $P = 0.32$). This may reflect differences in experimental variation between transgenic plants and in vitro assays, glucosinolate turnover in vivo, or enzyme function in vitro versus in vivo. Finally, we mutated Gly¹³⁴ and Pro⁵³⁶ in *BCMA2* (to *BCMA1/3* residues Leu and Lys, respectively; henceforth G134L and P536K) to assay their effect on catalytic activity on valine and isoleucine. Either mutation, or both together, caused increased activity toward valine ($P = 0.0361$ to $P = 0.0004$; Fig. 3B and table S14).

The tertiary structures of eukaryotic cytochrome P450 proteins are highly conserved despite substantial divergence in their primary structures (19, 20). We predicted the structure

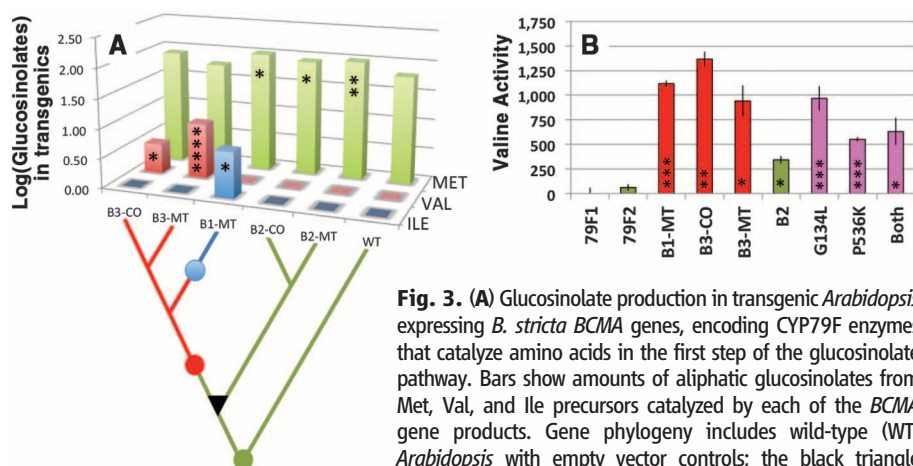


Fig. 3. (A) Glucosinolate production in transgenic *Arabidopsis* expressing *B. stricta* *BCMA* genes, encoding CYP79F enzymes that catalyze amino acids in the first step of the glucosinolate pathway. Bars show amounts of aliphatic glucosinolates from Met, Val, and Ile precursors catalyzed by each of the *BCMA* gene products. Gene phylogeny includes wild-type (WT) *Arabidopsis* with empty vector controls; the black triangle identifies the gene duplication in *Boechera*, a red circle shows

the origin of branched-chain amino acid catalysis, and a blue circle indicates elevated Ile activity. Abbreviations: B1, *BCMA1*; B2, *BCMA2*; B3, *BCMA3*, with alleles from Colorado (CO) or Montana (MT). $N = 130$ independent transgenic lines. **(B)** In vitro enzyme activity levels (nmol of product per nmol of enzyme per minute) relative to controls; error bars denote SE. Labels indicate CYP79F enzymes from *Arabidopsis* and *BCMA1*, *BCMA2*, and *BCMA3* from *Boechera*, with alleles from Colorado or Montana. *BCMA2* alleles encode identical proteins, so one allele was assayed. *BCMA2* (green) retains the ancestral MET activity and was engineered to change G134L, P536K, or both (pink). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

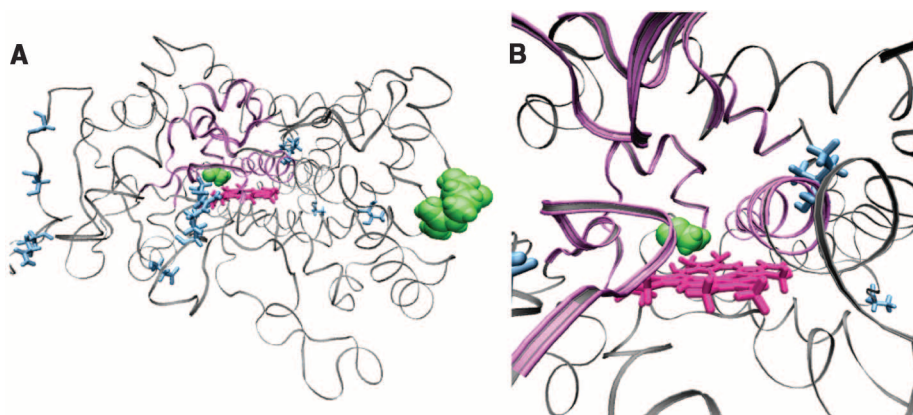


Fig. 4. (A) Homology model of *BCMA2* with the substrate-binding cleft above the heme group (magenta) with putative substrate recognition regions in purple. Amino acid changes G134L and P536K (green) show statistical evidence for accelerated protein evolution and alter catalytic function when changed by site-directed mutagenesis. Other mutations with statistical evidence of accelerated evolution (in blue) are not addressed in this study. The location of amino acid 529, which aligns with the last resolved residue in the CYP1A2 crystal structure, is colored because subsequent amino acids 530 to 540 cannot be accurately modeled. **(B)** Close-up view of substrate-binding cleft with mutation G134L residing just above the heme.

of BCMA2 (Fig. 4) and visualized the locations of variations in the BCMA1 and BCMA3 proteins that might explain their altered catalytic functions. G134L, one of the two residues showing evidence for accelerated molecular evolution, occurs in substrate recognition site SRS1 near the heme (Fig. 4) and is predicted to alter the catalytic site space in the region closest to the heme. The other, P536K, occurs five amino acids upstream from their C termini and is predicted to alter electrostatic interactions of this flexible tail region. Mapping of the two positions varying between the BCMA3-MT and BCMA3-CO alleles indicates that Val¹⁴⁸ → Leu occurs in a region potentially affecting interactions with electron transfer partners, and that Met²⁶⁸ → Val occurs in a SRS3 region predicted to affect the volume of the upper catalytic site and/or substrate access (fig. S6). However, determining the biochemical effects of these changes is beyond the scope of this study.

We have shown how the *BCMA* QTL affects plant chemistry and insect resistance, and thus fitness, in a quantitative manner. In *Boechea*, the *BCMA2* locus retains ancestral activity and synteny, whereas *BCMA1* and *BCMA3* have evolved novel catalytic activity. The resulting polymorphic Met-GS and BC-GS show heterogeneous effects on host plant resistance against diverse enemies across a range of environments. In the Montana population, homozygotes at *BCMA* produce BC-GS and show greater resistance to damage by a diverse community of herbivores (tables S4 and S6). Further evidence that these compounds

have environment-dependent consequences comes from transgenic *Arabidopsis*, where BC-GS cause increased resistance to the pathogen *Erwinia carotovora* (6), and from other herbivores, where BC-GS cause increased susceptibility to *Trichoplusia ni* (10). However, *BCMA* has no effect on insect damage in Colorado (tables S4 and S6), where other loci control resistance (table S6). On the basis of this study, we conclude that heterogeneous responses to diverse biotic interactions in the context of selection by herbivores likely contribute to the genetic diversity of *BCMA*.

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Acknowledgments: We thank R. Colautti, K. Donohue, M. Feder, T. Pendergast, M. Rauscher, C. Rushworth, A. Shumate, M. Wagner, J. Willis, and two anonymous reviewers for helpful comments. K. Springer, E. Ballweg, M. Cameron, K. Chu, S. Hurst, V. Cousins, K. Dales, R. Doll, J. Lutkenhaus, M. Mitchell-Olds, S. Mitchell-Olds, E. Raskin, J. Rivera, L. Saucier, M. Wagner, and T. Weiss-Lehman helped in lab and field. N. Wicks and Bitterroot National Forest allowed us to work on their property. We thank the Heald family for support and hospitality. Supported by NIH grants R01-GM086496 (T.M.-O.) and R01-GM079530 (M.A.S.), NSF grant EF-0723447 (T.M.-O.); NSF dissertation grants 1011167 (C.O.-M.) and 1110445 (C.-R.L.); and a Netherlands Organization for Scientific Research (NWO) Ecogenomics grant (M.E.S.). Data in the supplementary materials are also available as GenBank accession nos. JX185680, JX185681, and BCMA JQ337904 to BCMA JQ337909. Data are deposited in the Dryad Repository: <http://dx.doi.org/10.5061/dryad.kc6m8>.

Supplementary Materials

www.sciencemag.org/cgi/content/full/337/6098/1081/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S8
Tables S1 to S20
References (21–55)

8 March 2012; accepted 19 June 2012
10.1126/science.1221636

Arbuscular Mycorrhizal Fungi Increase Organic Carbon Decomposition Under Elevated CO₂

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The extent to which terrestrial ecosystems can sequester carbon to mitigate climate change is a matter of debate. The stimulation of arbuscular mycorrhizal fungi (AMF) by elevated atmospheric carbon dioxide (CO₂) has been assumed to be a major mechanism facilitating soil carbon sequestration by increasing carbon inputs to soil and by protecting organic carbon from decomposition via aggregation. We present evidence from four independent microcosm and field experiments demonstrating that CO₂ enhancement of AMF results in considerable soil carbon losses. Our findings challenge the assumption that AMF protect against degradation of organic carbon in soil and raise questions about the current prediction of terrestrial ecosystem carbon balance under future climate-change scenarios.

Arbuscular mycorrhizal fungi (AMF), which form associations with roots of ~80% of land plant species, obtain carbon (C) from their host plants in return for mineral nutrients (1, 2). AMF utilize a large proportion (up to 20%) of net plant photosynthates under ambient atmospheric CO₂ (aCO₂) (3, 4), deposit slow cycling organic compounds such as chitin

and glomalin (1, 5), and protect organic matter from microbial attack by promoting soil aggregation (6). AMF thus play a critical role in the global C cycle. Atmospheric CO₂ enrichment often increases plant photosynthate allocation to AMF and stimulates the growth of AMF (3, 7–9), leading to a proposition that global soils may sequester more C through mycorrhizal symbioses

under future scenarios of elevated CO₂ (eCO₂) (3, 5, 7–12). This hypothesis, however, does not consider the effect of AMF on decomposition under eCO₂. Indeed, AMF growth can result in enhanced decomposition of complex organic material and alter plant N uptake (13–15).

We conducted four independent but complementary experiments to investigate how CO₂ stimulation of AMF affects organic C decomposition in soil and the subsequent N dynamics in the plant-soil system by combining dual ¹³C/¹⁵N labeling and hyphae-ingrowth techniques (16). We first ascertained the effect of eCO₂ [main plot, *n* = 4; ambient at 380 versus elevated at 580 parts per million by volume (ppmv)] and N addition (subplot; control at 0 versus added at 5 g N m⁻²) on

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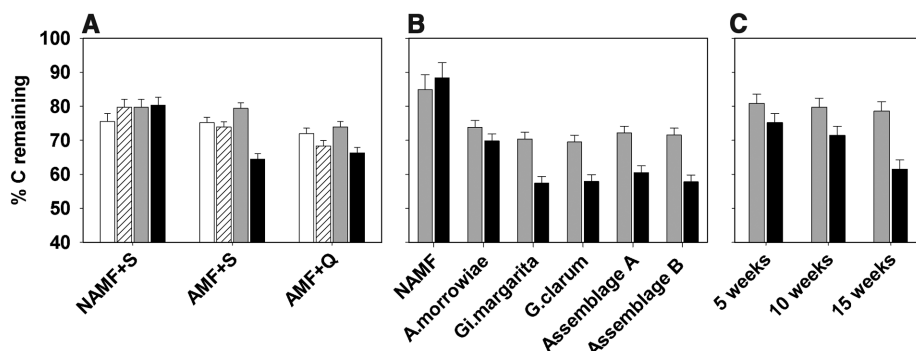


Fig. 1. The effect of arbuscular mycorrhizal fungi on organic C decomposition. **(A)** C remaining (%) within hyphae-ingrowth bags after 10 weeks of incubation under different CO₂ and N concentrations. +S and +Q refer to autoclaved sandy loam soil (S) and quartz sand (Q) in hyphae-ingrowth bags, respectively. Blank and gray bars denote ambient CO₂ without and with added N, respectively; hatched and black bars denote elevated CO₂ without and with added N, respectively. Data shown (means $\pm$ SEM) are based on the fitted mixed model. The main effects of N, and CO₂ $\times$ N and CO₂ $\times$ N $\times$ AMF interactions were not significant ($P > 0.05$). **(B)** and **(C)** C remaining (%) within hyphae-ingrowth cores after 10 weeks of incubation under different CO₂ and AMF species treatments (B) and within hyphae-ingrowth bags after 5, 10, and 15 weeks of incubation under different CO₂ concentrations in the field (C). Full AMF species name and assemblage composition are in table S1. Gray bars, ambient CO₂; black bars, elevated CO₂. Data shown (means $\pm$ SEM) are based on the fitted mixed model.

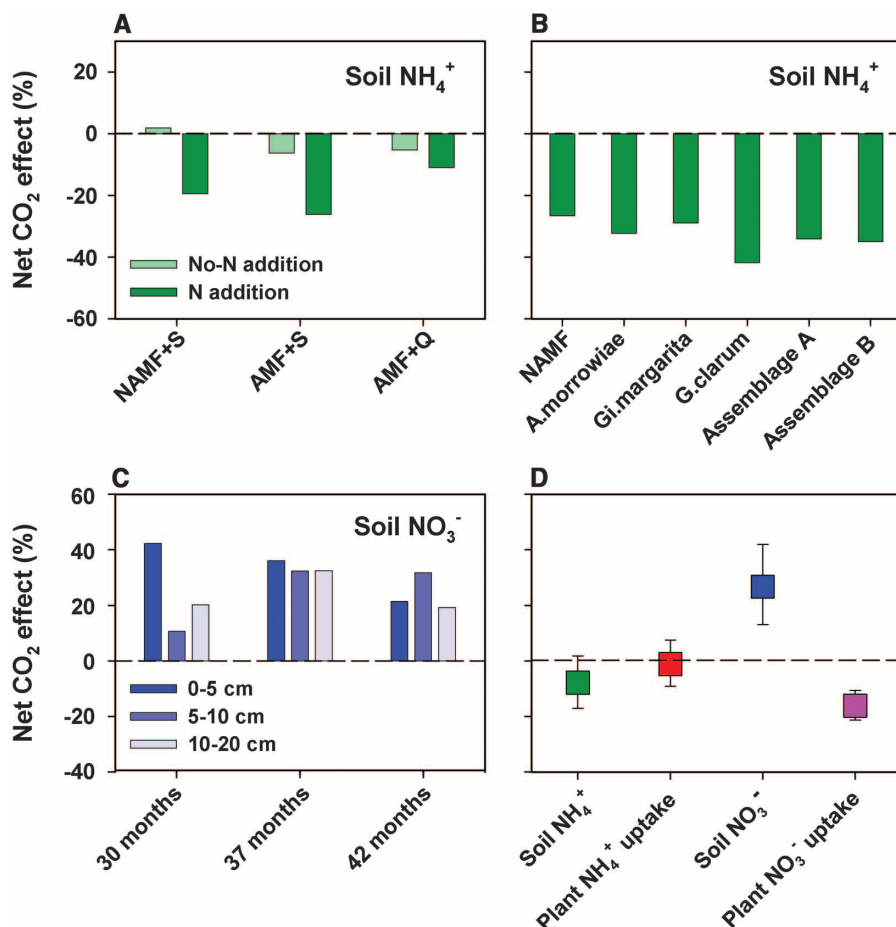


Fig. 2. Differential CO₂ effects on soil ammonium (NH₄⁺) and nitrate (NO₃⁻) and on plant NH₄⁺ and NO₃⁻ uptake. **(A)** to **(C)** Net CO₂ effect (%) on soil NH₄⁺ under different AMF and N concentrations (A) and different AMF species and assemblages (B) in microcosms, and on soil NO₃⁻ of three soil layers in the field (C). **(D)** A meta-analysis of net CO₂ effects (%) on soil NH₄⁺ ($n = 44$) and NO₃⁻ ($n = 30$), and on plant NH₄⁺ ($n = 71$) and NO₃⁻ ($n = 61$) uptake. Error bars, 95% confidence intervals. The elevated CO₂ effect on a response variable was considered significant if the 95% confidence interval did not overlap with 0.

mycorrhizal mediation of decomposition in a N-poor soil, using a model mycorrhizal plant community consisting of AMF growing on roots of *Avena fatua* (14) in microcosms (fig. S1). The high levels of CO₂ and N used in our experiment correspond to projected atmospheric CO₂ concentrations and N deposition rates in North America during the 21st century (17). We chose *A. fatua*, an annual C₃ grass native to Eurasia, because it has invaded many temperate grasslands and is considered one of the worst weeds in agricultural fields in North America.

After incubation for 10 weeks, AMF enhanced decomposition within hyphae-ingrowth bags ($P < 0.001$, Fig. 1A; also see ¹³C in fig. S2A). eCO₂ had no impact on total soil C in the absence of AMF (NAMF) ($P > 0.1$, Fig. 1A), but significantly reduced it by 9% in the presence of AMF ($P < 0.01$, Fig. 1A; see ¹³C in fig. S2A), consistent with the CO₂ stimulation of AMF infection of plant roots ($P < 0.05$, fig. S3A). Notably, the CO₂ effect on AMF-mediated decomposition mainly occurred under the N amendment, with a reduction in total C in hyphae-ingrowth bags of 19% in soil (AMF+S) and 10% in quartz sand (AMF+Q) (Fig. 1A; see ¹³C in fig. S2A).

Emerging evidence shows that AMF species may differ in their capabilities in acquiring N from decomposing residues (13). However, it is unknown whether the nature of AMF species or communities influences the CO₂ effect on residue decomposition. We investigated the effect of three individual AMF species and two AMF assemblages (subplot) on residue decomposition with their host plant *A. fatua* exposed to two atmospheric CO₂ levels (main plot, $n = 4$; 380 versus 580 ppmv) (16). One AMF assemblage consisted of three species and the other a total of eight species (table S1).

AMF enhanced decomposition in hyphae-ingrowth cores in comparison with the NAMF ($P < 0.001$, Fig. 1B; see ¹³C in fig. S2B), particularly under eCO₂. Across five AMF treatments, eCO₂ on average increased AMF infection of plant roots by 28% ($P < 0.05$, fig. S3B) and reduced total C by 15% within hyphae-ingrowth cores ($P < 0.05$, Fig. 1B; see ¹³C in fig. S2B). The magnitude of the CO₂ effect on decomposition differed among the three individual AMF species ($P < 0.05$), with the high effect found for both *Gigaspora margarita* and *Glomus clarum* and the low for *Acaulospora morrowiae*, but was comparable between the two AMF assemblages ($P > 0.1$). Taken together, these microcosm experiments indicate that CO₂ stimulation of AMF in general enhances organic C decomposition in soils with low N availability.

We also conducted a field study to examine the AMF effect on decomposition in a long-term CO₂ (380 versus 560 ppmv) and O₃ [20 versus 60 parts per billion by volume (ppbv)] experiment (2×2 factorial, $n = 4$) in a no-till wheat-soybean system (16, 18). We initiated the long-term experiment in May 2005 and carried out the decomposition study in the wheat season of 2008.

There were no significant O_3 or $CO_2 \times O_3$ effects on any soil microbial parameter (e.g., biomass C and N, fungi/bacteria ratio, and heterotrophic respiration) (18), AMF biomass and infection of roots, or organic C decomposition within hyphae- and root-ingrowth bags ($P > 0.05$). However, eCO_2 significantly increased both AMF colonization of fine roots collected from root-ingrowth bags ($P < 0.001$, fig. S3C) and the external AMF biomass as indexed by the biomarker fatty acid 16:1 ω 5c in the bulk soil ($P < 0.05$, fig. S3D). Concurrently, eCO_2 significantly increased total C losses within hyphae-ingrowth bags across the three sampling points ($P < 0.01$, Fig. 1C; see ^{13}C in fig. S2C). The instantaneous fractional loss rates for C ($k = 1 - X_t/X_0$, where X_t and X_0 are the organic C content at time t and time 0, respectively) induced by the hyphae-ingrowth effect under eCO_2 were 29, 41, and 80% higher than those under aCO_2 , respectively, at weeks 5, 10, and 15 (Fig. 1C), indicating that the CO_2 effect on AMF-mediated decomposition did not diminish over time.

To examine whether CO_2 enhancement of AMF-mediated decomposition was accompanied with increased plant uptake of N released from decomposing residues, we determined ^{15}N both in plants and hyphae-ingrowth bags and cores. eCO_2 substantially reduced the total ^{15}N within hyphae-ingrowth bags and cores in the presence of AMF in all three experiments (fig. S4) and increased AMF-mediated plant ^{15}N uptake in the microcosms (fig. S5). These results provide direct evidence of CO_2 enhancement of mycorrhizal N transfer from decomposing organic material to host plants.

We also examined the effect of eCO_2 on soil available N pools [ammonium (NH_4^+) and nitrate (NO_3^-)]. In microcosms where N was limiting and AMF were present, eCO_2 reduced soil NH_4^+

in both experiments ($P < 0.01$, Fig. 2A; $P < 0.05$, Fig. 2B), but did not affect levels of soil NO_3^- ($P > 0.1$, fig. S6D; $P > 0.1$, fig. S6E). In the field where soil N was ample (mainly NO_3^- , fig. S6F), eCO_2 did not affect soil NH_4^+ ($P > 0.1$ for each of three soil layers, fig. S6C) but significantly increased both potential N mineralization (18) and soil NO_3^- ($P < 0.05$ for each of three soil layers, Fig. 2C). These results suggest that eCO_2 may differentially affect plant acquisition of soil NH_4^+ and NO_3^- .

We subsequently conducted a meta-analysis (16) of 38 studies that quantified the concentrations of soil NH_4^+ and NO_3^- and/or the capacity of plant use of NH_4^+ and NO_3^- under eCO_2 (table S2). These studies encompassed more than 58 species of crop, grass, and tree species (16). eCO_2 reduced the capacity of plant NO_3^- use by 16.2% and increased soil NO_3^- by 26.7% (Fig. 2D). By contrast, it had no impact on the capacity of plants to use NH_4^+ but decreased soil NH_4^+ by 7.9% (Fig. 2D). These differential CO_2 effects on soil NH_4^+ and NO_3^- agreed with our results and were consistent qualitatively with recent discoveries of eCO_2 effects on plant N utilization (19, 20). Together, these results suggest that plants under eCO_2 may have to rely more on soil NH_4^+ for N nutrition, and a high demand for NH_4^+ may play a major role in mediating the AMF effect on organic C decomposition.

If CO_2 -induced high-plant demand for NH_4^+ is a primary driver in mycorrhizally mediated decomposition, high soil NH_4^+ may partially offset this effect. To test this possibility, we assessed the effect of AMF on decomposition by manipulating soil N transformations with a nitrifi-

cation inhibitor (dicyandiamide) (21) in our long-term field CO_2 and O_3 study in the wheat season of 2011 (16). Dicyandiamide had no effect on plant growth and AMF infection of roots ($P > 0.1$). In the no-dicyandiamide control, eCO_2 significantly increased AMF-mediated decomposition ($P < 0.05$, Fig. 3), consistent with the previous field experiment (Fig. 1C). In the dicyandiamide treatment, however, eCO_2 did not affect organic C decomposition in the hyphae-ingrowth bag ($P > 0.1$, Fig. 3), indicating that the nitrification inhibitor largely offset the impact of eCO_2 on AMF-mediated organic C decomposition. These results provide supporting evidence that enhanced plant demand for soil NH_4^+ may be the primary driver for CO_2 enhancement of AMF-mediated decomposition.

Based on this set of investigations, we therefore propose that eCO_2 enhancement of plant N demand prompts plants to invest more C and energy to structures (mainly roots and their associated mycorrhizae) that best garner NH_4^+ from soil (22), while stimulating NH_4^+ release from organic materials and reducing NH_4^+ substrate for nitrification (Fig. 4). Two unique AMF properties enable host plants to compete better against nitrifying microbes for NH_4^+ in the fine, discrete decomposing hotspots: (i) external AMF hyphae are at least two orders of magnitude longer and three orders of magnitude thinner than roots (1, 15) and can exploit a much larger soil volume and finer soil microsites; and (ii) AMF possess a special N transfer pathway (22, 23) that can transport soil N from external to internal hyphae and to their hosts preferentially as NH_4^+ with minimal C loss (23). Because AMF generally

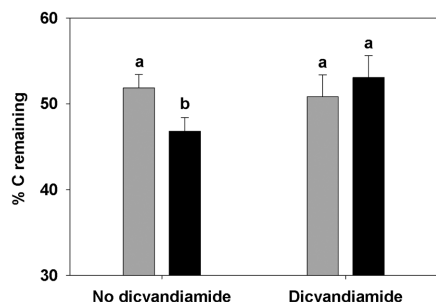
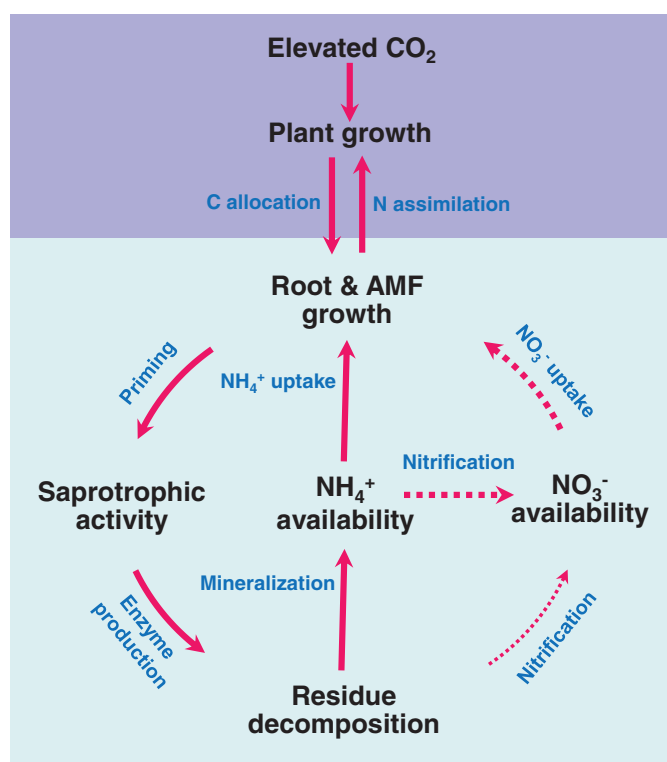


Fig. 3. A nitrification inhibitor (dicyandiamide) offset the CO_2 effect on organic C decomposition within hyphae-ingrowth bags after 10 weeks of incubation in the field. Gray bars, ambient CO_2 ; black bars, elevated CO_2 . Data shown (means $\pm$ SEM) are based on the fitted mixed model. The letters a and b represent a significant difference between two CO_2 levels under the no dicyandiamide treatment. The main O_3 effect and the $CO_2 \times O_3$ interaction were not significant in both dicyandiamide and no-dicyandiamide addition treatments ($P > 0.05$).

Fig. 4. A conceptual framework of AMF-mediated decomposition driven by CO_2 enhancement of plant N acquisition. CO_2 enhancement of AMF primes residue decomposition and ammonium (NH_4^+) release and optimizes NH_4^+ acquisition while reducing nitrification. CO_2 inhibition of nitrate (NO_3^-) photo-assimilation constrains the capacity of plant NO_3^- uptake, prompting plants to rely more on the AMF-mediated pathway of NH_4^+ (and possibly some simple organic N compounds) acquisition. Solid and dashed arrows represent positive and negative CO_2 effects, respectively.



lack saprotrophic capability (1), CO₂ enhancement of AMF for N scavenging likely increases decomposition by stimulating (i.e., priming) saprotrophs in soil through three potential mechanisms. First, AMF likely grow preferentially toward (15), and thus facilitate saprotrophs' access to, new organic patches (24). Second, AMF slowly release labile C for saprotrophs at relatively low concentrations (3), likely engendering a larger priming effect on decomposition than roots (fig. S7) (25–27). And third, rapid removal of newly released NH₄⁺ by AMF likely releases saprotrophs from metabolic repression (28).

Our findings indicate that CO₂ enhancement of AMF may alter terrestrial ecosystem C dynamics by stimulating decomposition of soil organic C in AMF-active zones. This effect will likely occur in its interplay with other controlling factors such as temperature and plant species composition (29). In many agro- or grassland ecosystems where AMF dominate (1), but no aboveground C pool with an annual incremental increase exists, CO₂ stimulation of AMF and organic C decomposition will mainly facilitate C turnover belowground, rather than ecosystem C sequestration (30). Even in forests with abundant AMF (e.g., tropical forests) (1), eCO₂ stimulation of AMF, although creating a transient C sink in plant biomass by facilitating N transfer from soil to plants and partially alleviating N limitation on plants (31), is likely to reduce the largest carbon stocks (soil C) in the system. Also, our results suggest that the form, rather than just the total amount, of soil N might play a major role in mediating belowground C turnover and plant N acquisition under eCO₂, thus offering a theoretical foundation for management of microbial N transformations in soil and plant N utilization to

facilitate ecosystem C sequestration under future CO₂ scenarios.

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Acknowledgments: We thank F. Chapin III, D. Coleman, Y. Luo, R. Miller, and M. Rillig for valuable comments; M. Gumpertz for advice on statistical analyses; J. Barton, W. Pursley, and E. Silva for technical assistance; and D. Watson and J. Morton for providing mycorrhizal inoculum. L.C. was primarily supported by a fellowship from U.S. Department of Agriculture (USDA)—Agricultural Research Service Plant Science Research Unit (Raleigh, NC) and in part by a USDA grant to S.H. (2009-35101-05351). S.H., L.C. and C.T. conceived experiments 1 to 4. K.O.B. and F.L.B. designed and maintained the long-term CO₂ and O₃ study. H.D.S. and T.W.R. contributed to design of experiments 1 and 2. L.C. performed experiments 1 to 3 and the meta-analysis study; and C.T., F.L.B., and L.Z. performed experiment 4. L.C. and S.H. analyzed the data and mainly wrote the manuscript with inputs from all coauthors. The data reported in this paper are deposited in the Dryad Repository (<http://dx.doi.org/10.5061/dryad.b7f53>).

Supplementary Materials

www.sciencemag.org/cgi/content/full/337/6098/1084/DC1
Materials and Methods
Figs. S1 to S7
Tables S1 and S2
References (32–79)

4 May 2012; accepted 10 July 2012
10.1126/science.1224304

How the Cucumber Tendril Coils and Overwinds

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The helical coiling of plant tendrils has fascinated scientists for centuries, yet the underlying mechanism remains elusive. Moreover, despite Darwin's widely accepted interpretation of coiled tendrils as soft springs, their mechanical behavior remains unknown. Our experiments on cucumber tendrils demonstrate that tendril coiling occurs via asymmetric contraction of an internal fiber ribbon of specialized cells. Under tension, both extracted fiber ribbons and old tendrils exhibit twistless overwinding rather than unwinding, with an initially soft response followed by strong strain-stiffening at large extensions. We explain this behavior using physical models of prestrained rubber strips, geometric arguments, and mathematical models of elastic filaments. Collectively, our study illuminates the origin of tendril coiling, quantifies Darwin's original proposal, and suggests designs for biomimetic twistless springs with tunable mechanical responses.

The transformation of a straight plant tendril into a helically coiled shape has inspired numerous studies since the 1800s (1–8), both from mechanistic and functional perspectives. Tendrils serve climbing plants by providing

a parasitic alternative to building independently stable structural supports, allowing the plant to wend its way to sunlight and numerous ecological niches (9). During climbing, an initially straight tendril first finds and attaches to a support

(fig. S1 and movie S1). Once tethered, the tendril coils by forming two oppositely handed helices connected by a “perversion” (Fig. 1, A and B), which was recognized by Darwin as a topological necessity given the clamped boundary conditions at each end of the tendril (3). This helical coiling axially shortens the tendril, hoisting the plant toward the attachment point (fig. S1 and movie S1).

Despite the long history of studying tendrils, the basic mechanism of tendril coiling has remained elusive. Historically, experimental studies of diverse tissues [reaction wood (10), hypocotyls (11), twining stems (12, 13), and leaves (14)] have addressed aspects of curvature generation, whereas

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theoretical treatments have incorporated intrinsic curvature or differential growth without addressing its origin or mechanical consequences (6, 15, 16). Recent studies of tendril anatomy (17, 18) have provided a new twist by revealing an interior layer of specialized cells similar to the stiff, lignified gelatinous fiber (g-fiber) cells found in reaction wood (19). These cells provide structural support in reaction wood via tissue morphosis driven by cell-wall lignification, water flux, and oriented stiff cellulose microfibrils. The presence of a similar ribbon-like strip of g-fiber cells in tendrils suggests that the coiling of the soft tendril tissue may be driven by the shaping of this stiff, internal “fiber ribbon” (18).

We investigated the role of the fiber ribbon during tendril coiling in both *Cucumis sativus* (cucumber) and *Echinocystis lobata* (wild cucumber) (20). The g-fiber cells, identified in wild cucumber by using xylan antibodies in (18), are easily distinguished as a band of morphologically differentiated cells consistently positioned along the inner side of the helical tendril that lignify during coiling (17, 18). In straight tendrils that have not yet attached to a support (Fig. 1A), a faint band of immature g-fiber cells is barely visible by using darkfield microscopy (Fig. 1B), with no ultraviolet (UV) illumination signature, indicating the absence of lignification (Fig. 1C). In coiled tendrils (Fig. 1D), g-fiber cells are clearly visible (Fig. 1E) and lignified (Fig. 1F). The fiber ribbon consists of two cell layers, with the ventral layer

on the inside of the helix showing increased lignification relative to the dorsal outer layer (Fig. 1, G and H), which is consistent with earlier observations of increased lignification on the stimulated side of the tendril (17, 18). When a fiber ribbon is extracted from the coiled tendril by using fungal carbohydrolases [Driselase (Sigma-Aldrich, St. Louis, MO)] to break down the nonlignified epidermal tendril tissue (20), it retains the helical morphology of a coiled tendril, and furthermore, lengthwise cuts do not change its shape (Fig. 1I and fig. S2).

These observations suggest that tendril coiling occurs via asymmetric contraction of the fiber ribbon; the ventral side shrinks longitudinally relative to the dorsal side, giving the fiber ribbon its intrinsic curvature. The asymmetric contraction may be generated by a variety of dorsiventral asymmetries, including the observed differential lignification (Fig. 1H), variations in cellulose microfibril orientation as in reaction wood, or differential water affinities. For example, because lignin is hydrophobic the ventral cells may expel more water during lignification, driving increased cell contraction. This would be consistent with observations of extracted fiber ribbons that passively shrink and coil even further when dried but regain their original shape when rehydrated (movie S2). Dehydrated tendrils also exhibit this behavior because they are dominated by the stiff fiber ribbon (movie S3). Together, these facts suggest that the biophysical mechanism for

tendril coiling is provided by the asymmetric contraction of the stiff fiber ribbon, whose resulting curvature is imposed on the surrounding soft tendril tissue. The perversions in a doubly supported tendril follow naturally from the topological constraint imposed by the prevention of twist at its ends.

To better understand the origin of curvature in fiber ribbons, we reconstituted the underlying mechanism using a physical model composed of two bonded, differentially prestrained silicone rubber sheets, similar to rubber models for shaping sheets (21–23). The first silicone sheet was uniaxially stretched, and an equally thick layer of silicone sealant was spread onto the stretched sheet. After the sealant was fully cured, thin strips were cut along the prestrained direction, yielding bilayer ribbons (Fig. 2A) with intrinsic curvature set by the relative prestrain, thickness, and stiffness of the two layers (fig. S3) (20). Like fiber ribbons, the initially straight physical models spontaneously form coiled configurations with two opposite-handed helices connected by a helical perversion (Fig. 2A, left).

However, there is an unexpected difference in mechanical behavior between the physical models and tendril fiber ribbons. When clamped at both ends and pulled axially, the physical model simply unwinds to its original uncoiled state (Fig. 2A and movie S4). In contrast, in fiber ribbons we observed a counterintuitive “overwinding” behavior in which the ribbon coils even further

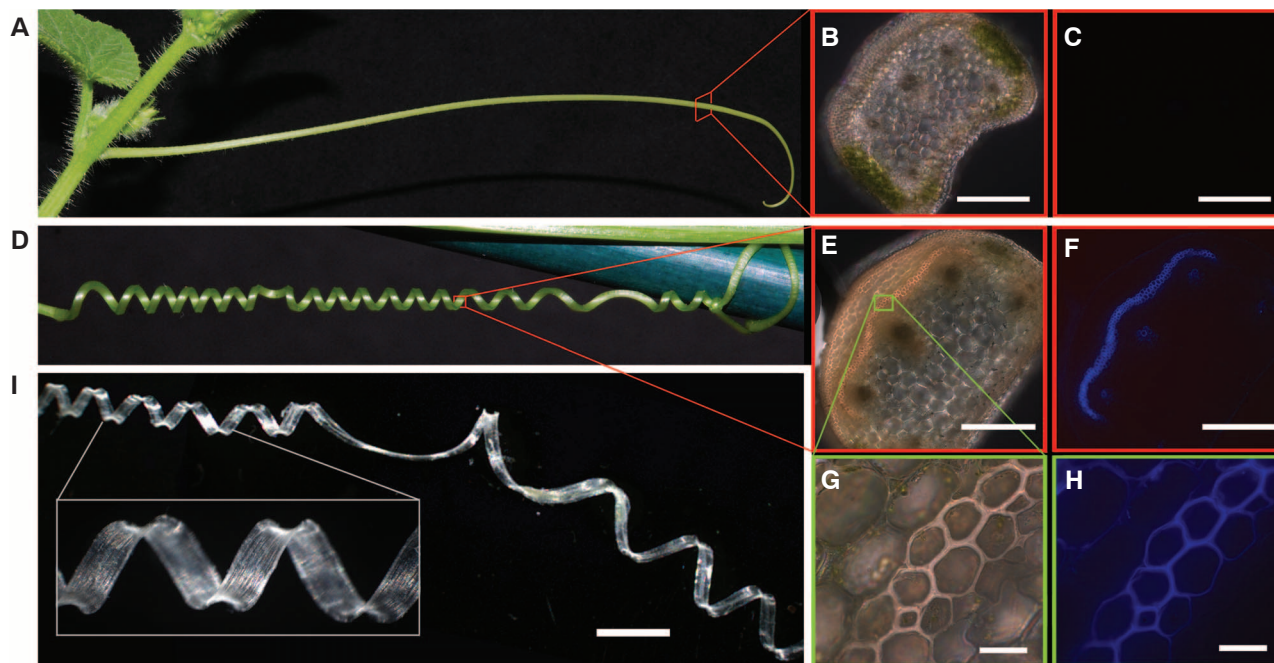


Fig. 1. Tendril coiling via asymmetric contraction. During coiling, a strip of specialized structural gelatinous fiber cells (the fiber ribbon) becomes lignified and contracts asymmetrically and longitudinally. (A to C) A straight tendril that has never coiled (A) lacks lignified g-fiber cells. In the tendril cross section, darkfield (B) and UV autofluorescence (C) show no lignin signal. (D to H) In coiled tendrils (D), the fully developed fiber ribbon consists of ~2 layers of highly lignified cells extending along the length of the tendril. In the tendril

cross section, darkfield (E) and UV autofluorescence (F) show strong lignification in the fiber ribbon. In (G) and (H), increased magnification reveals that ventral cells (top left) are more lignified than dorsal cells. (I) The extracted fiber ribbon retains the helical morphology of the coiled tendril. (Inset) Higher magnification shows the orientation of g-fiber cells along the fiber ribbon. Scale bars, (B) and (C) 0.5 mm, (E) and (F) 100 μ m, (G) and (H) 10 μ m, (I) 1 mm.

when pulled, adding turns on both sides of the perversion (Fig. 2A, right, and movie S5). Eventually though, under high enough tension the fiber ribbon unwinds, returning to a flat, uncoiled state as expected (movie S5).

Inspired by our observations of asymmetric lignification in fiber ribbons, which suggest that the inner layer is less extensible, we added a relatively inextensible fabric ribbon to the inside of a coiled physical model. To mimic lignified

cells that resist compression, we added an incompressible copper wire to the exterior of the helix. The internal fabric ribbon prevents elongation, whereas the external copper wire prevents contraction. Together, these modifications increase

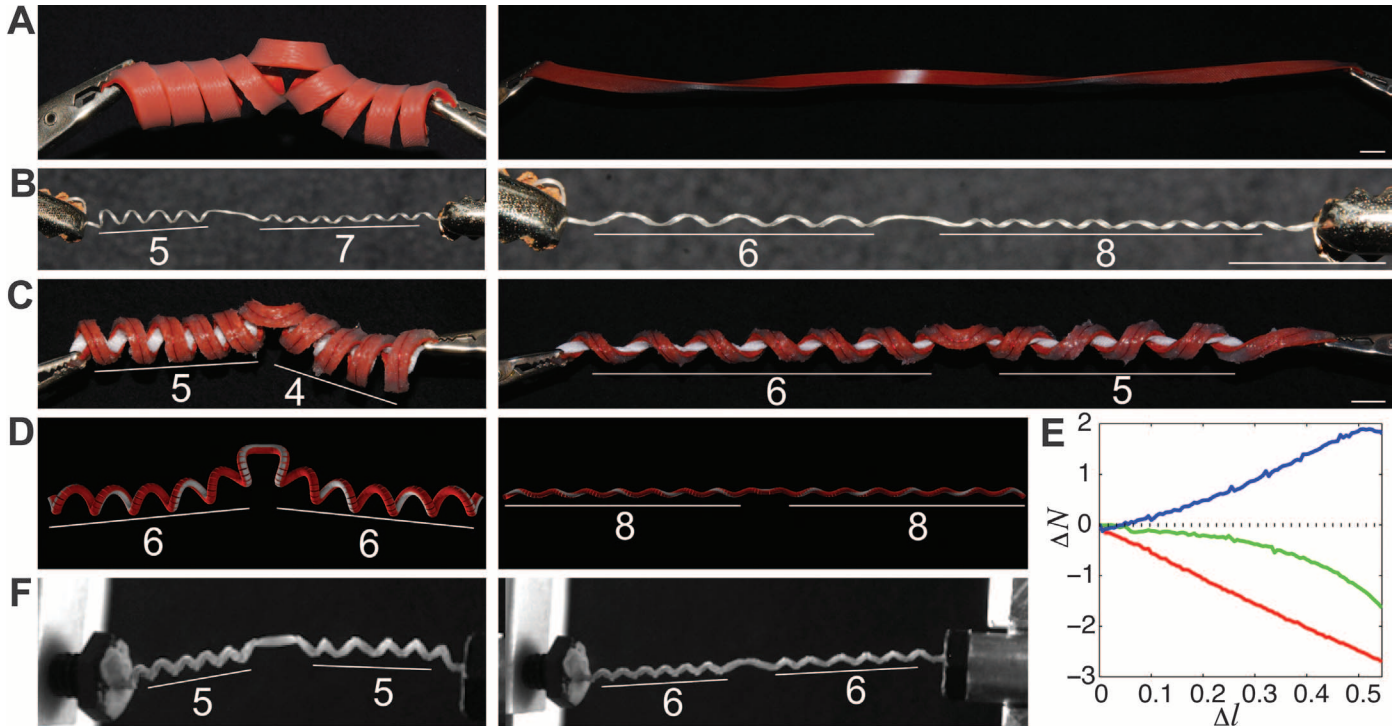
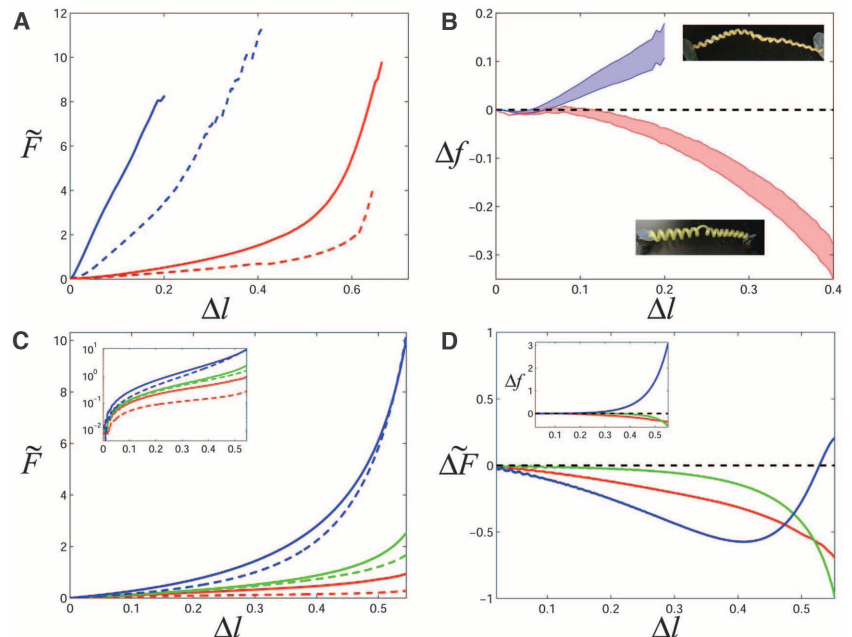


Fig. 2. Twistless springs unwinding and overwinding. (A) A silicone twistless spring with lower bending stiffness B than twisting stiffness C unwinds when pulled, returning to its original flat shape. (B) When a fiber ribbon is pulled, it initially overwinds, adding one extra turn to each side of the perversion (number of turns are indicated in white). (C) Overwinding is induced in the silicone model by adding a relatively inextensible (under tension) fabric ribbon to the interior of the helix and an inextensible (under compression) copper wire to the exterior.

Together, these increase the ratio B/C . (D) When $B/C > 1$, numerical simulations of elastic helical filaments recapitulate this overwinding behavior, which is consistent with physical and biological experiments. (E) Change in the number of turns in each helix ΔN is plotted versus scaled displacement Δl for B/C values 1/5 (red), 1 (green), and 5 (blue). Overwinding becomes more pronounced with increasing B/C . (F) Overwinding is also observed in old tendrils, which have dried and flattened into a ribbon-like shape with $B/C > 1$. Scale bars, 1 cm.

Fig. 3. Mechanical consequences of overwinding. (A and B) Force extension curves for one young tendril that does not overwind (red curves) and one old tendril that exhibits substantial overwinding (blue curves). Each tendril was separated into a segment containing the helical perversion (dotted curves indicate perverted) and a segment with no perversion (solid curves indicate clamped). The dimensionless force $\tilde{F}$ is plotted against the scaled displacement Δl (detailed definitions are available in the supplementary materials) in (A). The difference in scaled force due to the helical perversion $\Delta f = f(\text{perverted}) - f(\text{clamped})$ is plotted against Δl in (B). The shaded range in (B) indicates variations in the fitted initial slope value. (C) Dimensionless force-extension curves are plotted for numerical filaments with B/C values 1/5 (red), 1 (green), 5 (blue). (Inset) Log-linear plot of the same data. (D) The difference in force $\Delta \tilde{F} = \tilde{F}(\text{perverted}) - \tilde{F}(\text{clamped})$ highlights the mechanical effect of the helical perversion. For $B < C$, the perversion always decreases the force needed to axially extend the filament; for $B > C$, the perversion initially decreases the force needed but eventually increases this necessary force at higher extensions. (Inset) Δf is plotted against Δl for direct comparison with the experimental data.



the model's effective bending stiffness relative to its twisting stiffness, fixing its intrinsic helix curvature while still allowing twist about its centerline (20). The modified model exhibited substantial overwinding (Fig. 2C and movie S6). Indeed, a single helix with infinite bending stiffness and fixed curvature cannot extend without its ends rotating. However, if one end may rotate, additional axial length can be accommodated by changing both pitch and radius to maintain constant curvature, resulting in additional helical turns (20). The perversion connecting helices of opposite handedness allows rotation and enables the addition of helical turns. By overwinding, each helix can thus geometrically accommodate axial extension without varying its curvature (fig. S4).

Of course, real tendril fiber ribbons have finite stretching and bending stiffness, and eventually at sufficiently high tensions, the helices unwind. To study overwinding in a fiber ribbon with finite bending and twisting stiffness, we modeled it mathematically as a filament composed of two equal-length, elastic helices of opposite handedness but identical intrinsic curvature k_0 and torsion w_0 , and uniform bending stiffness B and twisting stiffness C , connected by a single helical perversion (Fig. 2D, left). When the filament, initially at equilibrium, is pulled apart at its clamped ends, deviations from equilibrium values of curvature and twist lead to variations in the filament's total energy (20). Minimizing the energy of the extended filament numerically (20), we determined the filament shape and position as a function of the applied tension (Fig. 2D, right). When $B/C < 1$, the filament unwinds on extension, but when $B/C > 1$, the filament overwinds (Fig. 2D and movies S7 and S8), and the number of additional turns ΔN increases with increasing B/C (Fig. 2E) (24). For comparison, for a helical spring with a circular cross-section made of an isotropic material, $B/C = 1 + \nu$, with Poisson ratio ν normally in the range $0 < \nu \leq 0.5$ so that typical springs exhibit minimal overwinding.

The observation of overwinding in fiber ribbons naturally leads to the question of whether entire tendrils also overwind. Whereas both young and old fiber ribbons always overwind, recently coiled, fully hydrated tendrils ("young" tendrils) do not overwind, but mature, dry tendrils ("old" tendrils) exhibit substantial overwinding (Fig. 2F and movies S9 and S10), and intermediate tendrils were variable in their overwinding behavior. The overwinding observed in old tendrils is likely due to the fact that as the tendril dries, the epidermal cells lose volume, and the tendril flattens down to a ribbon like shape similar to the internal fiber ribbon, so that $B/C > 1$.

To investigate the mechanical and functional consequences of overwinding, we measured the force required to axially stretch tendrils using a custom force measurement setup (20). Force-extension curves measured for a total of 20 tendrils show a variety of mechanical responses; in

Fig. 3, we plot the dimensionless force $\tilde{F}$ against the scaled displacement Δl (detailed definitions of Δl and $\tilde{F}$ are in the supplementary text) for the two most extreme cases, a young tendril (red) and an old tendril (blue). For each, we show the results for a segment containing the perversion (Fig. 3; dotted curves indicate "perverted"), and another for a segment without it (Fig. 3; solid curves indicate "clamped"). In the young tendril, the perverted segment is always softer than the clamped segment (Fig. 3A). In contrast, the perverted segment of the old tendril is initially softer than the clamped segment but becomes stiffer at large extensions. Plotting the difference $\Delta f = f(\text{perverted}) - f(\text{clamped})$, where the scaled force f is obtained by dividing each force curve by its own initial slope (Fig. 3B), we see that for the young tendril in which no overwinding occurs, Δf is always negative, indicating that the perversion consistently decreases the force necessary to stretch the tendril relative to the clamped case. However, in the old, overwinding tendril the perversion actually increases the force needed to stretch the tendril as Δl increases.

To quantify the behaviors bounded by these two extreme tendril measurements, we also calculated force-extension curves using our mathematical models. The dimensionless force-extension curves for filaments with $B/C = 1/5$ (red), 1 (green), and 5 (blue) are shown in Fig. 3C. Similar to the behavior of the young tendril, in the filament with $B/C = 1/5$ (no overwinding), the presence of the perversion decreases the stiffness of the system—the force needed to axially extend the filament. However, the force response qualitatively changes when $B/C \gtrsim 3$, and the filament exhibits substantial overwinding. As in the old tendril, initially the perversion decreases the force needed to stretch the filament, but at large extensions, the perversion actually increases the force needed; the differential stiffness of the system is non-monotonic. Indeed, we observed that the difference $\Delta \tilde{F} = \tilde{F}(\text{perverted}) - \tilde{F}(\text{clamped})$ is always negative for filaments with $B < C$, whereas in overwinding filaments with large B/C values, $\Delta \tilde{F}$ transitions to positive values at large extensions (Fig. 3D). Thus, in overwinding filaments a helical perversion initially softens the force response but eventually stiffens the filament relative to the clamped case, which is a behavior qualitatively different from earlier theoretical studies (6, 16), in which overwinding was not observed in the range of B/C values studied. The difference in scaled force Δf shown in the Fig. 3D inset is consistent with experimental observations (Fig. 3B), indicating that the unusual force-extension behavior shown in Fig. 3D explains the extremes observed in the two tendrils.

Collectively, our observations raise questions at an evolutionary level about the ubiquity of this mechanism in other tendril-bearing species and at a mechanical level about the functional principles of these soft twistless springs. Preliminary studies of *Passiflora* tendrils reveal a band of g-fibers, suggesting a similar coiling mechanism (fig. S5);

however, both young and old coiled *Passiflora* tendrils exhibit overwinding (fig. S5 and movie S11). Although Cucurbitaceae and Passifloraceae are from the same phylogenetic clade, their tendrils have evolved independently (25), inviting future comparative studies between species as well as investigations of subcellular processes regulating asymmetric contraction. Functionally, the combination of mechanical asymmetry, helical perversions, and large ratios of bending to twisting stiffness creates an autoadaptive springy tendril, one that is initially soft because it can overwind and then stiffens strongly when deformed further. Darwin himself wrote that "the tendril strikes some object, and quickly curls round ... contracts into a spire, dragging up the stem, and forming an excellent spring" (3). Our study illuminates and quantifies this proposal biophysically while suggesting biomimetic variants of the humble helical spring.

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Acknowledgments: This research was supported by funding from the MacArthur Foundation, the Wyss Institute,

and the Kavli Institute. S.J.G., J.R.P., and L.M. designed the study. S.J.G., J.R.P., A.G.M., and L.M. conducted the research. S.J.G. and J.R.P. performed the biological and biophysical experiments. S.J.G., A.G.M., and L.M. handled biophysical theory. S.J.G., J.R.P., A.G.M., and L.M. contributed analytical tools and reagents and analyzed data. S.J.G., J.R.P., and L.M. wrote the paper.

Harvard University has filed a patent application relating to a tunable, twistless overwinding spring based on the results of this study.

Supplementary Materials

www.sciencemag.org/cgi/content/full/337/6098/1087/DC1
Materials and Methods

Supplementary Text

Figs. S1 to S5

References (26–29)

Movies S1 to S11

13 April 2012; accepted 19 June 2012

10.1126/science.1223304

A Single Progenitor Population Switches Behavior to Maintain and Repair Esophageal Epithelium

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Diseases of the esophageal epithelium (EE), such as reflux esophagitis and cancer, are rising in incidence. Despite this, the cellular behaviors underlying EE homeostasis and repair remain controversial. Here, we show that in mice, EE is maintained by a single population of cells that divide stochastically to generate proliferating and differentiating daughters with equal probability. In response to challenge with all-trans retinoic acid (atRA), the balance of daughter cell fate is unaltered, but the rate of cell division increases. However, after wounding, cells reversibly switch to producing an excess of proliferating daughters until the wound has closed. Such fate-switching enables a single progenitor population to both maintain and repair tissue without the need for a “reserve” slow-cycling stem cell pool.

Murine esophageal epithelium (EE) consists of layers of keratinocytes. This tissue lacks structures such as crypts or glands that form stem cell niches in other epithelia (Fig. 1, A and B) (1–5). Proliferation is confined to cells in the basal layer (6). On commitment to terminal differentiation, basal cells exit the cell cycle and subsequently migrate to the tissue surface from which they are shed. Early studies suggested that all proliferating cells were functionally equivalent, but recent reports propose that a discrete population of slow-cycling stem cells is responsible for both maintenance and wound healing (7–11). This controversy and the importance of EE in disease motivated us to resolve the proliferative cell behavior in homeostatic EE and in tissue challenged by systemic treatment with the vitamin A metabolite all-trans retinoic acid (atRA) or acute local wounding (12, 13).

To investigate cell division rates in EE, we used a transgenic label-retaining cell (LRC) assay (Fig. 1C) (1, 14, 15). Doxycycline (DOX) induction of the fusion protein Histone-2B enhanced green fluorescent protein (HGFP) expression in

Rosa26^{M2rtTA}/TetO-HGFP mice resulted in nuclear fluorescent labeling throughout the EE (Fig. 1D and fig. S1A). When DOX is withdrawn, HGFP is diluted by cell division, leaving 0.4% basal layer cells (561 out of 140,000) retaining label after a 4-week chase (Fig. 1E and fig. S1B). Three-dimensional imaging showed that these LRCs had smaller nuclei than the surrounding keratinocytes and did not stain for the basal keratinocyte marker Keratin14 (0 out of 561 LRCs) (fig. S1, C and D). The stem cell markers CD34

and *Lgr5* were also undetectable in LRCs or other cells (figs. S2 and S3) (2, 4, 10, 16). However, 99.9% (2457 out of 2459) of LRCs were positive for the pan leukocyte marker CD45 (Fig. 1E, inset), comprising a mixture of Langerhans cells and lymphocytes (fig. S1, E and F). These findings lead to the unexpected conclusion that, unlike tissues such as the epidermis, there are no slow-cycling or quiescent epithelial stem cells in EE (1, 17). Indeed, HGFP dilution in basal cells was strikingly homogeneous, suggesting that all cells divide at a similar rate of about twice per week (fig. S1G).

Although epithelial cells have the same rate of division, they may still differ in their ability to generate cycling and differentiated progeny. We therefore used inducible cre-lox-based genetic marking to investigate whether the proliferating cell population is heterogeneous and to quantify cell behavior (18, 19). The fate of single-cell-derived clones was tracked in cohorts of adult Ahcre^{ERT} R26^{REYFP/wt} mice at multiple time points over a year after induction, during which period EE was homeostatic (Fig. 2A and fig. S4). Crucially, analysis of the composition of clones at 1 year showed that they were representative of unlabeled cells (fig. S5). Over the time course, clone number decreased through differentiation, whereas the size of the remaining clones progressively increased (Fig. 2, B and C). Although variation in labeling efficiency limits the accuracy with which the proportion of labeled cells can be estimated, within statistical error,

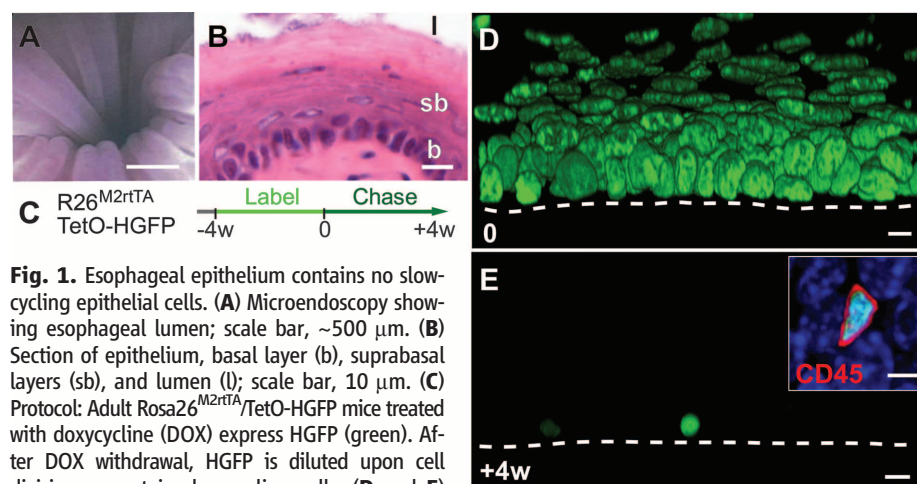


Fig. 1. Esophageal epithelium contains no slow-cycling epithelial cells. (A) Microendoscopy showing esophageal lumen; scale bar, ~500 μ m. (B) Section of epithelium, basal layer (b), suprabasal layers (sb), and lumen (l); scale bar, 10 μ m. (C) Protocol: Adult Rosa26^{M2rtTA}/TetO-HGFP mice treated with doxycycline (DOX) express HGFP (green). After DOX withdrawal, HGFP is diluted upon cell division, except in slow-cycling cells. (D and E) Rendered confocal z stacks, showing HGFP (green) at time 0 (D) and after 4-week chase (E). Scale bar, 10 μ m. Dashed line indicates basement membrane. Inset shows CD45 (red) staining in HGFP-retaining cell at 4 weeks. 4',6-diamidino-2-phenylindole (DAPI), blue; scale bar, 5 μ m.

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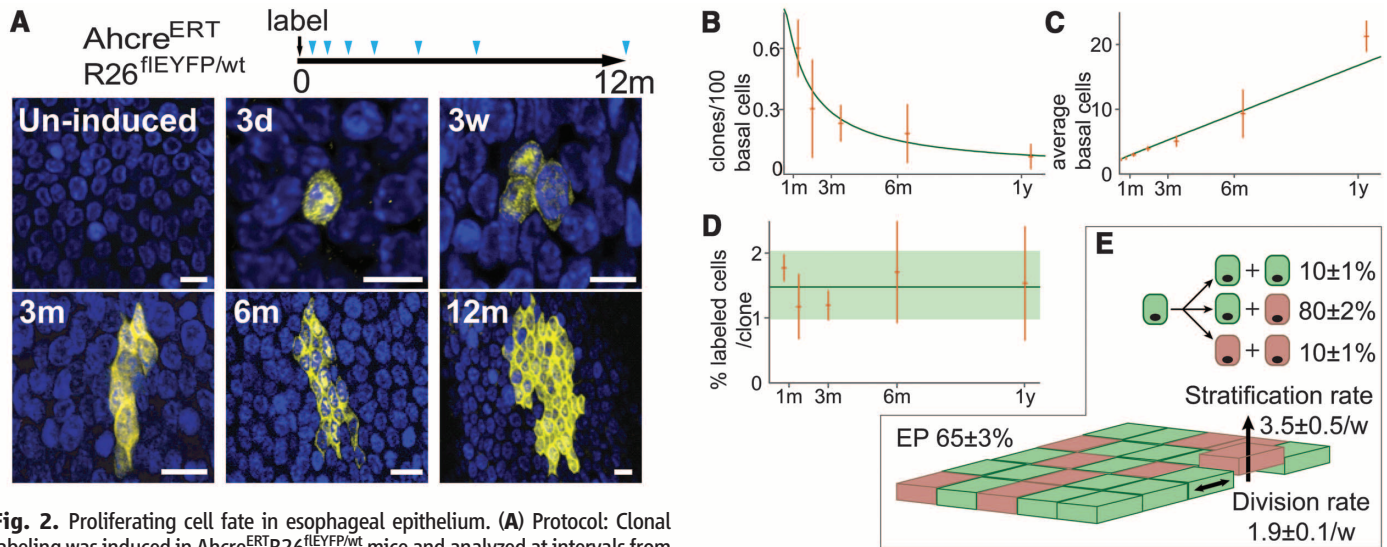


Fig. 2. Proliferating cell fate in esophageal epithelium. **(A)** Protocol: Clonal labeling was induced in $Ahcre^{ERT}R26^{fIEYFP/wt}$ mice and analyzed at intervals from 3 days to 1 year (triangles). Images are rendered confocal z stacks of the basal layer showing typical clones at times indicated. Enhanced yellow fluorescent protein (EYFP), yellow; DAPI, blue. Scale bars, 10 μ m. **(B to D)** Clone quantification. **(B and C)** Clone density and average clone size (basal cells). Observed values (orange) with error bars (mean $\pm$ SEM); green curves show predictions of model **(E)**. **(D)** Average percentage of labeled basal cells at indicated time points (orange); error bars indicate mean $\pm$ SEM. Green line and shading show average

and SEM across all time points. **(E)** Cell fate in EE. Basal layer comprises 65% functionally equivalent EP (green, dividing at a rate of 1.9/week, consistent with the rate of dilution of HGFP (fig. S1G) and 35% postmitotic cells (pink), which stratify (arrow) at a rate of 3.5/week. Ten percent of EP divisions generate two EP daughters, 10% two differentiated daughters, and 80% one of each fate. Values are optimal fits with 95% plausible intervals.

this proportion remains constant, which is consistent with the labeled population being in homeostasis (Fig. 2D).

Notably, the average size of persisting clones increased linearly with time, and their size distribution acquired long-term scaling behavior, a hallmark of a single functionally equivalent population of cells dividing at the same rate (Fig. 2C and fig. S6, A, B, and E) (18–21). Studies of interfollicular epidermis (IFE) revealed that this pattern of clonal evolution was consistent with progenitors dividing stochastically to generate differentiated and cycling daughters with equal probability (18, 19). By implementing a Bayesian inference analysis, we showed that the entire data set conforms to the IFE paradigm (Fig. 2E; fig. S6, C to E; and supplementary theory). We conclude that esophageal progenitors (EP) are functionally equivalent.

The observation of similar progenitor behavior in EE and epidermis, derived from endoderm and ectoderm, respectively, argues that squamous epithelia share a common mechanism of homeostasis irrespective of their developmental origin. However, EP behavior differs from that of crypt stem cells in the endoderm-derived intestinal epithelium, where stochastic fate is a result of competition for limited niche space (16, 22).

Unlike progenitors in other tissues, such as the epidermis, EP are not supported by a discrete slow-cycling stem cell population (1). This raises the intriguing question of how the tissue responds to stress or injury. To investigate this issue, we subjected EE to a tissue-wide challenge in the form of atRA treatment and to acute local excisional wounding.

To determine the effects of atRA, we selected a dose that induced a “hyperproliferative” response (fig. S7A) and then used quantitative lineage tracing to define the changes in cell behavior (23, 24). Mice were treated for 9 days, clonal labeling was induced, and treatment then continued for a further 21 days, when clone size was scored (Fig. 3A). Bayesian analysis revealed that the rates of EP proliferation and differentiated cell stratification had approximately doubled. There was a small but statistically significant decrease in the proportion of proliferative cells but, critically, no significant change in the proportions of symmetric and asymmetric divisions, which indicated that the treated tissue was homeostatic (Fig. 3, B to D). To evaluate this finding, we used a second experimental schedule in which clonal labeling was induced before atRA treatment. The values of parameters determined in the first experiment accurately predicted the number of basal cells per clone on completion of the second protocol (fig. S7, B to D). We conclude that during atRA treatment, EP establish a new homeostatic state.

To investigate the repair of EP after wounding, we developed microendoscopic biopsy of mouse esophagus (fig. S8F). Biopsy produced a typical epithelial wound response (25, 26). Cells immediately next to the defect formed a migrating front (mf) in which there was minimal proliferation, surrounded by a proliferative zone (pz) in which cell division was dramatically increased (Fig. 4B and fig. S8D). We used three different protocols to analyze cell behavior (Fig. 4). First, we examined clonally labeled EP in $Ahcre^{ERT}R26^{fIEYFP/wt}$ mice induced 1 week before biopsy

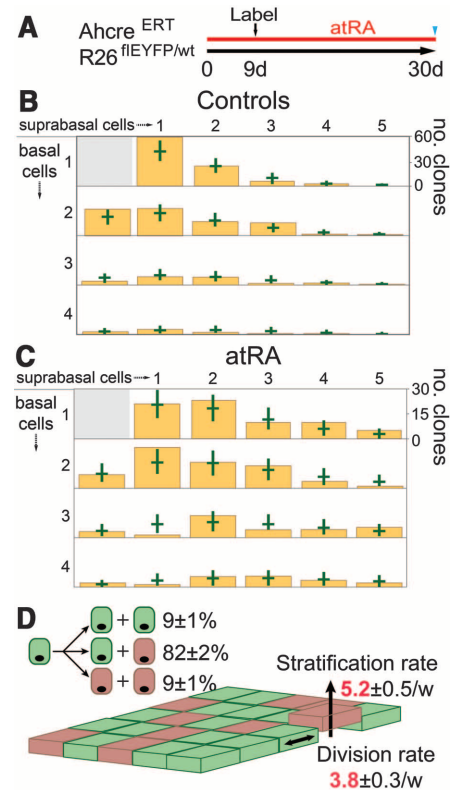


Fig. 3. All-trans retinoic acid (atRA) treatment of EE. **(A)** Protocol (see text). **(B and C)** Size distribution of multicellular clones containing at least one basal cell in control [(B), 307 clones] and atRA-treated [(C), 300 clones] EE. Green bars indicate 95% plausible fit to models in Figs. 2E (control) and 3D (atRA). **(D)** Optimal fit during atRA treatment; proliferation and differentiation rates (red) increase compared with control.

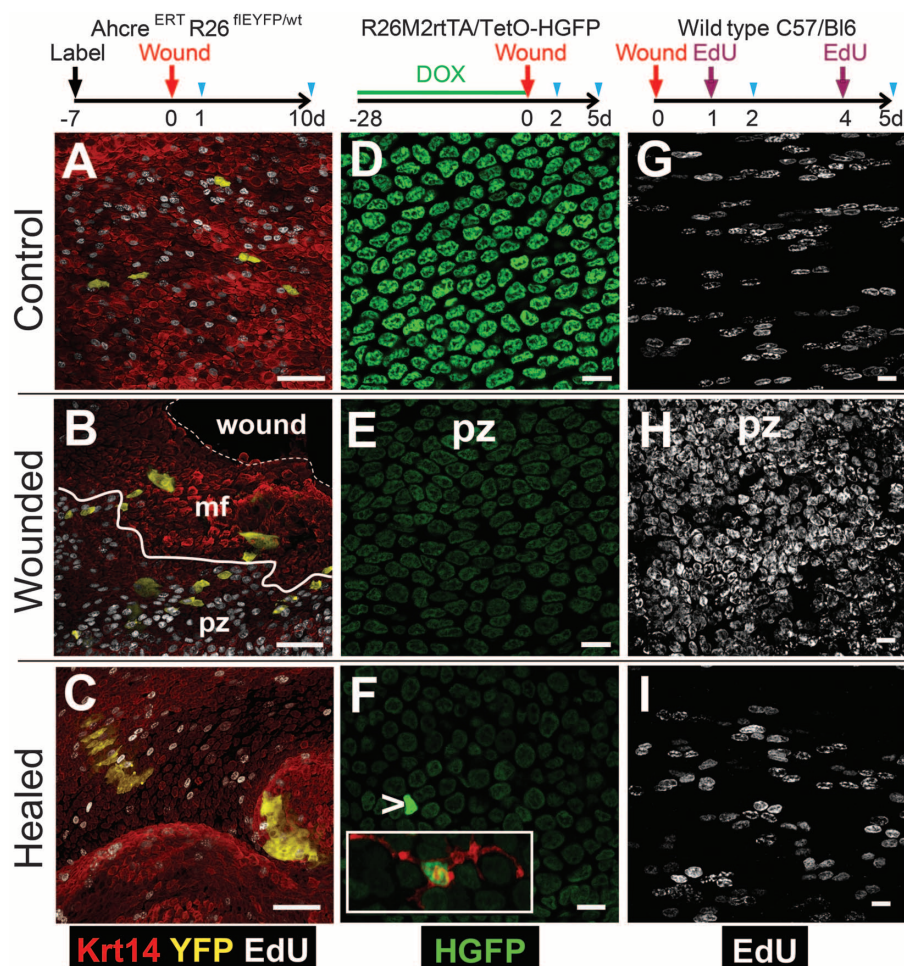


Fig. 4. Response of EP to wounding. Cartoons show protocols; blue triangles indicate sampling. (A to C) Wounding of clonally labeled mice. Confocal z stacks, 1 (B) or 10 (C) days after biopsy. Solid line shows pz-mf boundary; dashed line shows wound margin. (A) Day 1 unwounded control. EYFP is yellow, keratin 14 (Krt14) red, and EdU grayscale. Scale bars, 50 μ m. (D to F) Dilution of HGFP. Confocal z stacks from unwounded control day 2 (D) and wounded mice at 2 (E) and 5 (F) days after biopsy, showing HGFP (green). Arrow indicates HGFP bright cell (overexposed to reveal remaining cells); such cells stain for CD45 (red, inset). Scale bars, 10 μ m. (G to I) Cell proliferation. Confocal z stacks from unwounded control at 2 days (G) and experimental mice at 2 (H) and 5 (I) days after biopsy stained for EdU (grayscale). Scale bars, 10 μ m.

(Fig. 4, A to C). Twenty-four hours after wounding, fragmented clones of labeled cells were seen, aligned toward the wound and spanning the pz and mf (Fig. 4, A and B, and fig. S8D). By 10 days, clones were evident in and around the repaired defect (Fig. 4C and fig. S8, A and E). These findings indicate that EP participate in tissue regeneration after wounding, a behavior recapitulated in explant culture, suggesting that active recruitment of immune cells is not essential for the switch in EP fate (fig. S9) (27, 28). To investigate the proportion of EP that participate in regeneration, we biopsied DOX-treated Rosa26^{M2rtTA}/TetO-HGFP mice (Fig. 4, D to F). HGFP was substantially and evenly diluted within the pz at 2 and 5 days after biopsy compared with controls but was retained outside the mf (Fig. 4, D to F; fig. S8B; and fig. S10, A and B). We conclude that there is

widespread mobilization of EP within the pz and that the recruited cells proliferate to a similar extent. In a complementary experiment, animals were injected with EdU (5-ethynyl-2'-deoxyuridine) 24 hours before culling, revealing extensive recruitment of cells into cycle in the pz at 2 days, which reverted to control levels at 5 days when the epithelial defect had closed (Fig. 4, G to I; fig. S8C; and fig. S10, C to F). This indicates that the switch in EP fate after wounding is reversible.

In summary, these results show that EE is both maintained and repaired by a single progenitor cell population capable of reversibly switching between homeostatic and regenerative behavior in response to injury. These findings may be reconciled with the reported proliferative heterogeneity of EE cells in vitro if only some EP cells switch into "wound mode" when placed into cul-

ture (10). The widespread participation of progenitors in tissue repair provides a rapid and robust mechanism of wound healing without an underpinning stem cell pool.

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Acknowledgments: We thank E. Choolun and the staff at ARES and CBS Cambridge for technical assistance; D. Winton and D. Adams (Cambridge) for mice; and M. Gonzalez (London) for the Geminin antibody. We acknowledge the support of the MRC, EPSRC (Engineering and Physical Sciences Research Council), the NC3Rs (National Centre for the Replacement, Refinement and Reduction of Animals in Research), the Wellcome Trust, Sidney Sussex College, Cambridge (D.P.D.), European Union Marie Curie Fellowship PIEF-LIF-2007-220016 (M.P.A.), the Royal College of Surgeons of England (A.R.), and Cambridge Cancer Centre (A.R.). This work uses methods included in the patent WO2009010725 (A2), a method of detecting altered behavior in a population of cells; inventors were P.H.J., B.D.S., and A.M.K.

Supplementary Materials

www.sciencemag.org/cgi/content/full/science.1218835/DC1
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References (29, 30)

6 January 2012; accepted 10 July 2012
Published online 19 July 2012;
10.1126/science.1218835

Identification of Small Molecule Activators of Cryptochrome

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Impairment of the circadian clock has been associated with numerous disorders, including metabolic disease. Although small molecules that modulate clock function might offer therapeutic approaches to such diseases, only a few compounds have been identified that selectively target core clock proteins. From an unbiased cell-based circadian phenotypic screen, we identified KL001, a small molecule that specifically interacts with cryptochrome (CRY). KL001 prevented ubiquitin-dependent degradation of CRY, resulting in lengthening of the circadian period. In combination with mathematical modeling, our studies using KL001 revealed that CRY1 and CRY2 share a similar functional role in the period regulation. Furthermore, KL001-mediated CRY stabilization inhibited glucagon-induced gluconeogenesis in primary hepatocytes. KL001 thus provides a tool to study the regulation of CRY-dependent physiology and aid development of clock-based therapeutics of diabetes.

The circadian clock is an intrinsic time-keeping mechanism that controls the daily rhythms of numerous physiological processes, such as sleep/wake behavior, body temperature, hormone secretion, and metabolism (1–3). Circadian rhythms are generated in a cell-autonomous manner through transcriptional regulatory networks of clock genes. In the core feedback loop, the transcription factors CLOCK and BMAL1 activate expression of *Period* (*Per1* and *Per2*) and *Cryptochrome* (*Cry1* and *Cry2*) genes. After translation and nuclear localization,

PER and CRY proteins inhibit CLOCK-BMAL1 function, resulting in rhythmic gene expression (1–3). Rate-limiting steps in many physiological pathways, including hepatic processes, are under the control of the circadian clock (1–3). The gluconeogenic genes *phosphoenol pyruvate carboxykinase* (*Pck1*) and *glucose 6-phosphatase* (*G6pc*) are controlled by CRY and the nuclear receptor REV-ERB (4–6).

Perturbations to clock function by genetic mutations or environmental factors (for example, shift work and jet lag) have been implicated in

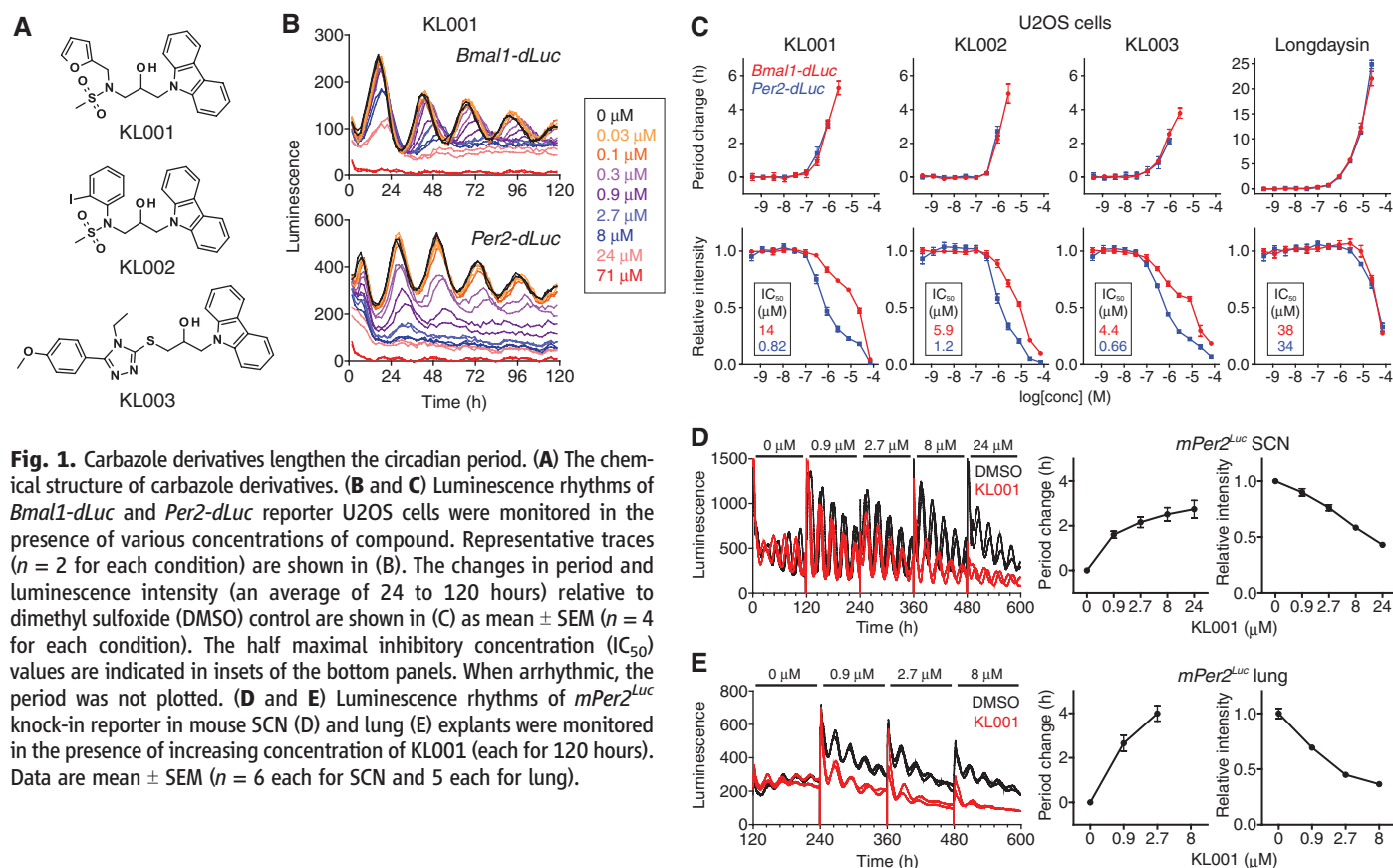
sleep disorders, cancer, and cardiovascular and metabolic diseases (1–3). Thus, identification of clock-modulating small molecules may prove useful for the treatment of circadian-related disorders. Through cell-based high-throughput chemical screening approaches, a number of compounds that affect circadian rhythms have been discovered, including casein kinase I (CKI) inhibitors such as longdaysin (7–11). Synthetic ligands for the nuclear receptors REV-ERB and ROR have also been used to regulate the clock and metabolism (12, 13). Here, we report the identification and characterization of a small molecule that specifically acts on CRY proteins and, as a result, regulates hepatic gluconeogenesis.

To identify small molecule modulators of the circadian clock, we analyzed the effect of a library of ~60,000 compounds on circadian rhythms

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of human osteosarcoma U2OS cell lines harboring a *Bmal1-dLuc* luciferase reporter (14). Among molecules that lengthened the period of luminescence rhythms, three carbazole derivatives (KL001 to KL003) (Fig. 1A) had pronounced effects. Continuous treatment with these compounds caused period lengthening and amplitude reduction in a dose-dependent manner in stable U2OS reporter cell lines harboring *Bmal1-dLuc* or *Per2-*

dLuc (Fig. 1, B and C, and fig. S1). Additionally, treatment of cells with these compounds lowered basal reporter activity in *Per2-dLuc* cells compared with that of *Bmal1-dLuc* cells, whereas longdaysin had equivalent effects on both reporter cells (Fig. 1, B and C, and fig. S1). High concentrations of KL001 (>50 μ M) exhibited cytotoxicity against U2OS cells (fig. S2). We further tested the effect of KL001 on transiently

transfected *Bmal1-dLuc* and *Per2-dLuc* reporters in mouse NIH-3T3 fibroblasts (fig. S3) and on a *mPer2^{Luc}* knock-in reporter (15) in explants of mouse suprachiasmatic nucleus (SCN) and lung (Fig. 1, D and E). KL001 caused dose-dependent lengthening of the period as well as signal reduction of *Per2* reporters at the transcription (*Per2-dLuc*) and protein (*mPer2^{Luc}*) levels in all assays. The compound had reduced potency in

Fig. 2. KL001 interacts with CRY1 and CRY2. (A) Agarose-conjugated KL001-linker compound was incubated with lysate of unsynchronized U2OS cells in the presence of various concentrations of free KL001 as a competitor. Bound proteins were identified by protein immunoblotting. Asterisk indicates nonspecific band. ppt, pellet. (B) Flag-tagged PER or CRY was transiently overexpressed in HEK293T cells, and lysates containing PER or a mixture of lysates containing PER and CRY (PER-CRY) were subjected to the pull-down assay. Bound proteins were detected with antibody to Flag. (C) Effects of KL001, KL002, KL004, and FAD on interaction of CRY1-Flag with KL001-agarose conjugate. (D) WT or FAD binding site mutant (D387N or N393D) of CRY1-Flag was subjected to the pull-down assay.

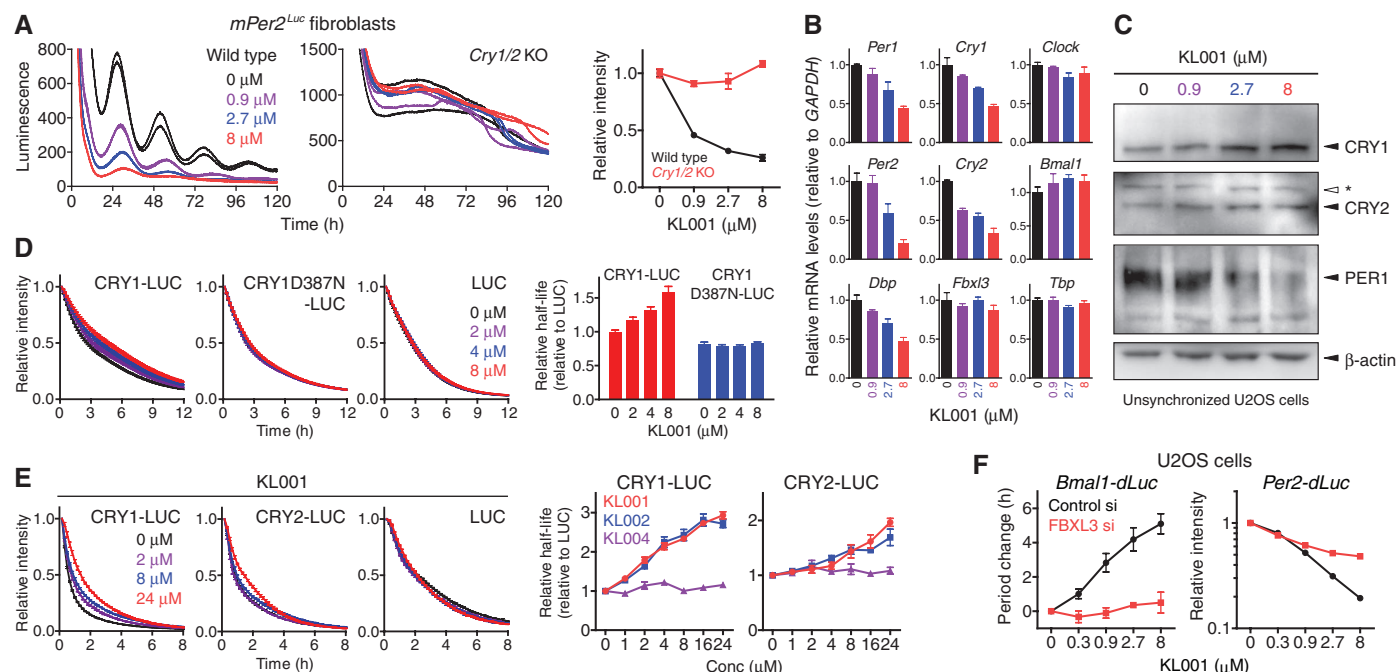
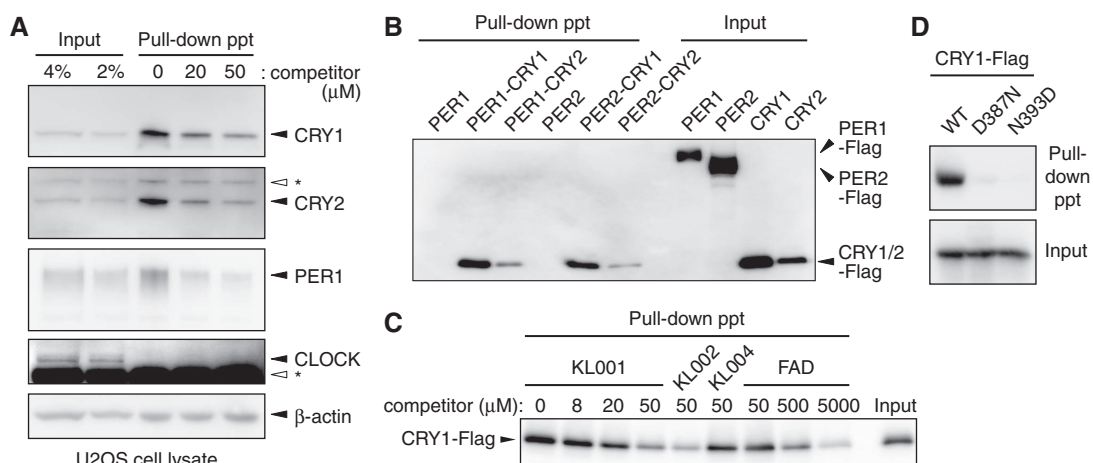


Fig. 3. KL001 stabilizes CRY proteins. (A) Effect of KL001 on *mPer2^{Luc}* knock-in reporter in *Cry1/2* double-knockout mouse fibroblasts. Data are mean $\pm$ SEM ($n = 4$ for each condition). (B and C) Confluent unsynchronized U2OS cells were treated with KL001 for 48 hours and then subjected to reverse transcription-quantitative polymerase chain reaction (RT-qPCR) (B; mean $\pm$ SEM, $n = 3$ for each condition) or protein immunoblot (C) analysis. (D) Luciferase-fused CRY1 (CRY1-LUC), its D387N mutant (CRY1D387N-LUC), or luciferase (LUC) was transiently overexpressed in HEK293T cells. The cells were treated with KL001 for 24 hours, and

then cycloheximide was added before luminescence recording. Profiles are shown by setting peak luminescence as 1 (left panels). Half-life of CRY1-LUC or CRY1D387N-LUC relative to LUC is shown by setting CRY1-LUC 0 μ M condition as 1 (right panel). Data are mean $\pm$ SEM ($n = 8$ for each condition). (E) Effects of KL001, KL002, and KL004 on CRY1 and CRY2 stability in HEK293 stable cell lines expressing CRY1-LUC, CRY2-LUC, or LUC. Data are mean $\pm$ SEM ($n = 4$ for each condition). (F) Effect of FBXL3 knockdown on the action of KL001 in *Bmal1-dLuc* or *Per2-dLuc* reporter U2OS cells. Data are mean $\pm$ SEM ($n = 4$ for each condition).

the SCN explant (Fig. 1D), suggesting a difference between SCN and peripheral clocks. Although period lengthening has been linked to the inhibition of casein kinase (CK) I δ , CKI α , or CK2 (9, 16), the carbazole derivatives did not affect their activities in vitro (fig. S4), suggesting an alternative mechanism of action.

To identify the molecular target(s) of KL001, we used an affinity-based proteomic approach. A limited structure-activity relationship study identified a derivative of KL001 with an ethylene glycol substituent at the methanesulfonyl position that maintained period lengthening activity (KL001-linker) (fig. S5A). We therefore prepared an agarose conjugate of KL001-linker and incubated it with U2OS cell lysate in the presence of 0, 20 or 50 μ M KL001. Proteins that bound to the affinity resin and were released in the presence of free KL001 were analyzed by liquid chromatography–tandem mass spectrometry. In two independent experiments, only CRY1 was identified as a candidate of KL001-binding protein (table S1). Protein immunoblotting with CRY1-specific antibody (5) (fig. S6) confirmed both the binding of CRY1 to the affinity resin and decreased binding in the presence of 20 and 50 μ M of free KL001 (Fig. 2A). We further used antibodies against other core clock proteins and detected interaction of the affinity resin with CRY2, and to a much lower extent PER1, but not CLOCK. β -actin showed nonspecific binding that was not displaced by free KL001 (Fig. 2A). In extracts of human

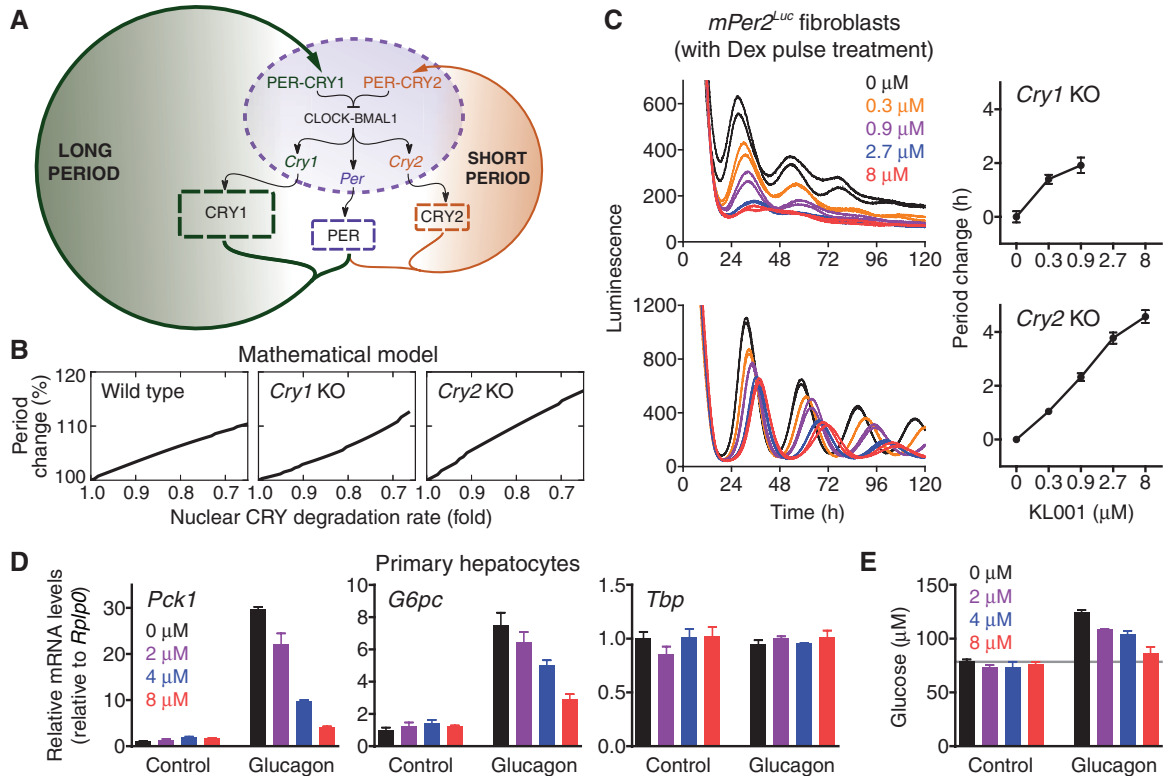
embryonic kidney (HEK) 293T cells transiently overexpressing Flag-tagged core clock proteins, the KL001-agarose conjugate interacted with CRY1 and CRY2, but not PER1, PER2, CLOCK, or BMAL1 (Fig. 2B and fig. S7). Purified CRY1 proteins also directly bound to the affinity resin (fig. S8). KL001 and KL002 similarly displaced CRY1 from the affinity resin (Fig. 2C), and KL004, an analog with a weak period effect (fig. S5B), blocked CRY1 binding less effectively (Fig. 2C). Flavin adenine dinucleotide (FAD), a cofactor of CRY family proteins (17), inhibited CRY1 interaction with the affinity resin when added in excess (500 to 5000 μ M) (Fig. 2C and fig. S9). Moreover, CRY1 proteins with mutations in the FAD binding sites (CRY1D387N and CRY1N393D) (17) interacted very weakly with the affinity resin (Fig. 2D). Thus, KL001 selectively interacts with CRY.

We analyzed the effect of KL001 on the *mPer2^{LUC}* knock-in reporter in *Cry*-deficient mouse fibroblasts. KL001-mediated reduction of the *mPer2^{LUC}* intensity in wild-type (WT) cells was abolished in the *Cry1/2* double-knockout cells (Fig. 3A). Similarly, small interfering RNA (siRNA)-mediated depletion of CRY1 and CRY2 diminished the KL001-dependent reduction of *Per2-dLuc* intensity in U2OS cells (fig. S10). Furthermore, a mutation of the CLOCK-BMAL1-binding site, the E2 enhancer element (18), abrogated the *Per2* reporter response to KL001 in an NIH-3T3 transient transfection assay (fig. S11). Thus, the com-

pound likely enhances the repressive activity of CRY on the *Per2* reporter in a CRY- and E2 enhancer-dependent manner.

Treatment of unsynchronized U2OS cells with KL001 reduced amounts of endogenous *Per2* mRNA in a dose-dependent manner and had almost no effect on *Bmal1* (Fig. 3B). Other CLOCK-BMAL1 target genes—*Per1*, *Cry1*, *Cry2*, and *Dbp*—exhibited a pattern of suppression similar to *Per2* (Fig. 3B), consistent with KL001 enhancing CRY. Although amounts of PER1 protein decreased in parallel with *Per1* mRNA expression after KL001 treatment, amounts of CRY1 and CRY2 did not correlate with their mRNA expression and were increased and sustained, respectively (Fig. 3C). Thus, KL001 may stabilize CRY proteins. We therefore analyzed the effect of KL001 on the half-life of CRY1 by transiently expressing a CRY1-luciferase fusion protein (CRY1-LUC) in HEK293T cells. Treatment of cells with KL001 led to a dose-dependent increase in the half-life of CRY1-LUC but did not affect the stability of the FAD binding site mutant CRY1D387N (Fig. 3D). In HEK293 stable cell lines expressing CRY1-LUC, CRY2-LUC, or LUC, treatment with KL001 and KL002 increased the half-life of CRY1 and CRY2, whereas the same concentrations of KL004 showed almost no effect (Fig. 3E and fig. S12). The effect of each compound on CRY stability is consistent with their effects on the circadian period (Fig. 1C and fig. S5B) and CRY1 interaction (Fig. 2C),

Fig. 4. Application of KL001 to define roles of CRY isoforms (A to C) and to control hepatic gluconeogenesis (D and E). (A) Scheme of the mathematical model consisting of the two parallel CRY feedback loops. (B) Effect of nuclear CRY stabilization on the period in WT, *Cry1* knockout and *Cry2* knockout cells in silico. (C) *Cry1* or *Cry2* knockout *mPer2^{LUC}* knock-in mouse fibroblasts were stimulated with dexamethasone (Dex) for 2 hours, and luminescence rhythms were monitored in the presence of KL001. Data are mean $\pm$ SEM ($n = 4$ for each condition). (D and E) Mouse primary hepatocytes were treated with KL001 for 18 hours and then stimulated with 10 nM glucagon for 2 hours (D, for RT-qPCR analysis) or 3 hours (E, for glucose assay). To measure glucose production, the cells were further incubated with glucose-free buffer containing 20 mM sodium lactate and 2 mM sodium pyruvate for 4 hours (E). Data are mean $\pm$ SEM ($n = 3$ for each condition).



connecting the compound-dependent CRY stabilization with period lengthening. Because CRY proteins are targets of an E3 ubiquitin ligase complex SCF^{FBXL3} and degraded through the ubiquitin-proteasome pathway (19–21), we tested the effect of KL001 on CRY1 ubiquitination in vitro in a lysate of HEK293T cells transiently overexpressing CRY1-Flag. The compound (50 μ M) inhibited ubiquitination of CRY1 and showed only a little effect on the CRY1D387N mutant (fig. S13). Moreover, siRNA-mediated depletion of FBXL3 in U2OS reporter cells diminished the effects of KL001 on the period and *Per2* reporter intensity without affecting long-day effects (Fig. 3F and fig. S14). These results indicate that KL001 inhibits FBXL3- and ubiquitin-dependent degradation of CRY proteins and further support the selectivity of the compound.

We then used KL001 in combination with mathematical modeling to explore how KL001-mediated CRY stabilization results in period lengthening and to define the roles of the seemingly redundant CRY isoforms in the clock mechanism. We constructed a simple mathematical model of the PER-CRY negative feedback loop (Fig. 4A and fig. S15A) (14). The model successfully reproduced period shortening and lengthening by dose-dependent knockdown of *Cry1* and *Cry2*, respectively (22) (fig. S15B), and also period shortening by stabilization of cytosolic CRY2 (23) (fig. S15C). For period lengthening by KL001-dependent CRY stabilization, the model predicted that the stabilization occurs in the nucleus (Fig. 4B, left panel, and fig. S15D). Indeed, amounts of CRY1 and CRY2 proteins were increased and sustained, respectively, in a nuclear fraction of unsynchronized U2OS cells after KL001 treatment, although amounts of PER1 were reduced (fig. S16). Furthermore, in silico stabilization of nuclear CRY2 in a *Cry1* knockout background and nuclear CRY1 in a *Cry2* knockout background both caused period lengthening (Fig. 4B, middle and right panels). Consistent with this prediction, continuous treatment with KL001 lengthened the period in both *Cry1* knockout and *Cry2* knockout fibroblasts in a dose-dependent manner (Fig. 4C and fig. S17, A and B). Similarly, the compound caused period lengthening in CRY1 knockdown and CRY2 knockdown U2OS cells (fig. S17C) and in SCN explants from *Cry1* knockout and *Cry2* knockout mice (fig. S17D). Thus, both CRY isoforms share a similar functional role in the period regulation, despite different free-running periods in their knockouts (Fig. 4A). With both CRY1 and CRY2 feedback loops intact, the nuclear CRY1/CRY2 ratio controls the period in a bidirectional manner; that is, more CRY1 causes longer periods and more CRY2 causes shorter periods (fig. S15, B and C).

In the liver, CRY proteins negatively regulate fasting hormone-induced transcription of the *Pck1* and *G6pc* genes, which encode rate-limiting enzymes of gluconeogenesis (4, 5). We therefore

tested the effect of KL001 on expression of these genes in mouse primary hepatocytes. KL001 repressed glucagon-dependent induction of *Pck1* and *G6pc* genes in a dose-dependent manner without affecting their basal expression (Fig. 4D). Consistent with this result, KL001 treatment repressed glucagon-mediated activation of glucose production (Fig. 4E). This repression was specific, because basal glucose production (Fig. 4E) and cellular lactate dehydrogenase activity (fig. S18) were unaffected. Altogether, our results demonstrate the potential of KL001 to control fasting hormone-induced gluconeogenesis. Given that human genome-wide association studies identified an association of the *CRY2* gene locus with fasting blood glucose concentrations and presentation of type 2 diabetes (24, 25), KL001 may provide the basis for a therapeutic approach for diabetes.

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Acknowledgments: We thank E. Peters, X. Liu, M. Garcia, C. Cho, and R. Glynn for assistance; C. Doherty for critical reading; and K. Lamia and J. Takahashi for reagents. This work was supported in part by grants from NIH (GM074868, MH051573, and GM085764 to S.A.K.; GM096873 to F.J.D.; and MH082945 to D.K.W.), Skaggs Institute for Chemical Biology (to P.G.S.), the U.S. Army Research Office (W911NF-09-0001 to F.J.D.), and a Department of Veterans Affairs Career Development Award (to D.K.W.). S.A.K. and P.G.S. serve on the Board of Reset Therapeutics and are paid consultants.

Supplementary Materials

www.sciencemag.org/cgi/content/full/science.1223710/DC1
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References (26–43)

23 April 2012; accepted 27 June 2012

Published online 12 July 2012;

10.1126/science.1223710

Extreme Bendability of DNA Less than 100 Base Pairs Long Revealed by Single-Molecule Cyclization

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The classical view of DNA posits that DNA must be stiff below the persistence length [<150 base pairs (bp)], but recent studies addressing this have yielded contradictory results. We developed a fluorescence-based, protein-free assay for studying the cyclization of single DNA molecules in real time. The assay samples the equilibrium population of a sharply bent, transient species that is entirely suppressed in single-molecule mechanical measurements and is biologically more relevant than the annealed species sampled in the traditional ligase-based assay. The looping rate has a weak length dependence between 67 and 106 bp that cannot be described by the worm-like chain model. Many biologically important protein-DNA interactions that involve looping and bending of DNA below 100 bp likely use this intrinsic bendability of DNA.

Bending and looping of lengths of DNA below 100 base pairs (bp) is ubiquitous in cellular processes such as regulated gene expression in bacteria and eukaryotes (1, 2), packaging of DNA in viral capsids, and DNA storage complexes in eukaryotes (3). Quantifying the intrinsic bendability of DNA at these bi-

ologically important length scales is essential for understanding DNA-protein interactions. According to a widely used approximation, DNA duplex is modeled as an elastic rod and its mechanical properties are described by the worm-like chain (WLC) model. Persistence length (l_p) is a measure of the bending rigidity of DNA; for a DNA

molecule that is several kilobasepair (kbp) or longer, l_p can be readily measured using single-molecule manipulation tools and is about 50 nm or 150 bp (4). In this framework, formation of DNA loops or sharp bends over length scales shorter than l_p incurs a large energetic cost that makes the probability of their spontaneous formation vanishingly small.

Many approaches have been developed to quantify and model the inherent flexibility and bendability of DNA at short length scales. In the cyclization assay, the ligase protein traps DNA molecules in the looped conformation, and then the looped and unlooped populations are separated based on their different gel mobility (5). Recent experiments using this assay and other techniques have challenged the classical picture of DNA as an elastic rod (6). Cyclization of DNA fragments of ~100 bp using the ligase assay yielded up to four orders of magnitude higher cyclizability (j factor) compared to the prediction of the WLC model (7). However, this extraordinary result was questioned and attributed to too high a ligase concentration used in the experiments (8). Small-angle x-ray scattering was used to measure end-to-end distance variations of short DNA fragments labeled with gold nanoparticles. These experiments suggested a cooperative stretching behavior over two helical turns (9); however, some aspects of the data are still paradoxical (10–12). Analysis of DNA images acquired using atomic force microscopy also deduced lower bending energies than WLC predicts (13). However, this method is indirect, is based on surface absorption of DNA molecules, and does not provide any dynamic information.

Contradictory and inconclusive results from these measurements call for a more direct approach to quantify flexibility of DNA on short length scales. In addition, most existing bulk approaches suffer from inherent limitations, such as limited range of physical conditions and formation of by-products other than monomer DNA circles, that limit their applicability to other systems. For example, due to nonspecific interactions of DNA and the ligase protein, the ligase-based assay may not be suitable for studying cyclization of very short DNA molecules (14). Moreover, looping events cannot be detected in real time using bulk techniques. Because of geometrical and technical limitations, single-molecule DNA-stretching approaches cannot be used to study the mechanics of very short DNA molecules. Even for a moderate length of DNA with several hundred bp, many corrections are required to account for the finite chain length and the boundary conditions (15). In addition, because of the relatively long persistence length of double-stranded

DNA (dsDNA), even 100 femto-Newton of force makes configurations with sharp local bending inaccessible. Therefore, DNA responses measured by mechanical stretching would not include any contribution from such sharply bent conformations even if they existed in a relaxed DNA.

We developed a cyclization assay, based on single-molecule fluorescence resonance energy transfer (smFRET) (16, 17), for directly monitoring the cyclization of single DNA molecules (Fig. 1A) (18). To avoid dimer formation during long-term observation, DNA molecules are immobilized on a polymer-coated surface through a biotin linker attached to a base at an internal DNA location. We avoided motifs such as A tracts, which are known to induce considerable intrinsic curvature (19). The DNA probe is a duplex with single-stranded extensions on both 5' ends. Each DNA molecule is labeled with Cy3 (donor) and Cy5 (acceptor) fluorophores at the 5' end of the strands. Single-stranded overhangs

are complementary so that hybridization will trap the DNA molecules in the looped state. In the unlooped state, the donor and acceptor are distant from each other and the molecules show zero FRET. Looping brings the dyes close to each other, and the DNA molecules exhibit a high FRET signal. Therefore, the looped state can be clearly distinguished from the unlooped state based on the FRET value and the relative intensities of donor and acceptor (Fig. 1B).

The experiment starts in a buffer without added ions in order to strongly favor the unlooped state. Introducing a buffer containing high concentrations of Na^+ or Mg^{2+} can stabilize the looped state. Depending on the lengths of the DNA duplex and the single-stranded overhangs, different behaviors were observed during the probing time window, typically ranging from less than 1 min to up to 4 hours. DNA molecules formed stable loops, showed dynamics between looped and unlooped conformations, or exhibited no looping events.

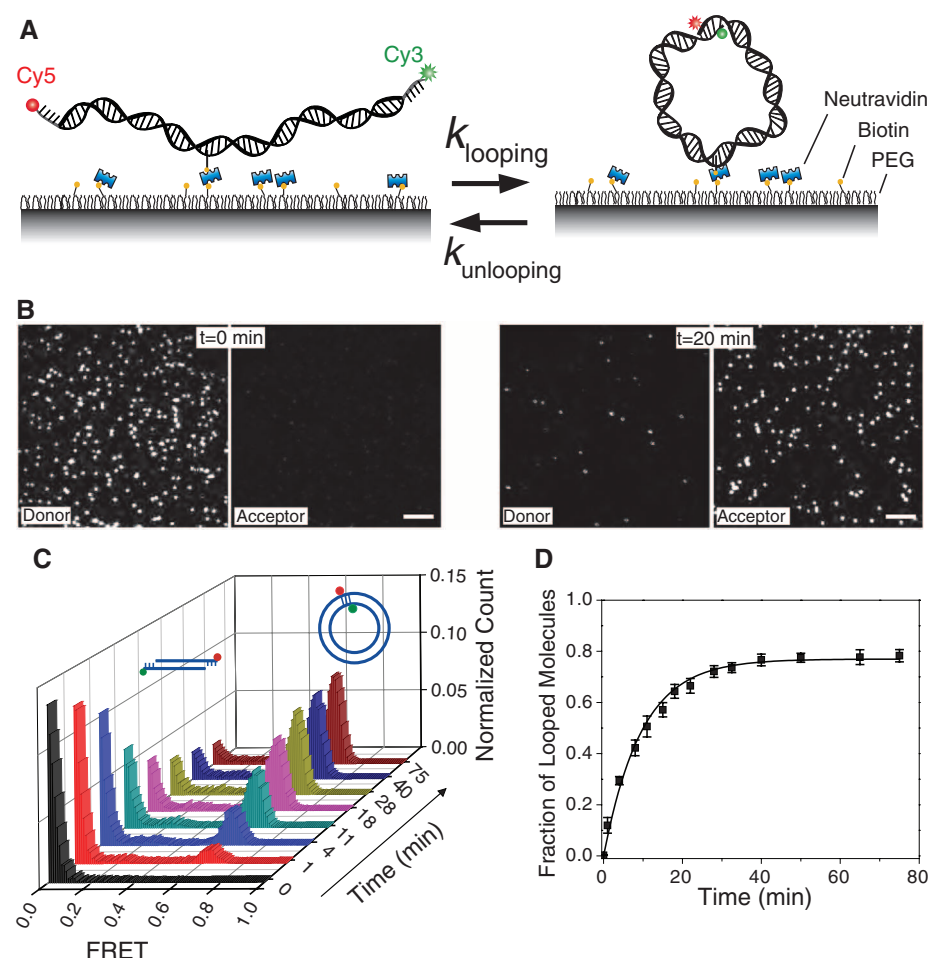


Fig. 1. (A) Donor (Cy3) and acceptor (Cy5) labeled DNA molecules were immobilized on the surface via biotin-neutravidin interaction. (B) Fluorescence images of single 91-bp DNA molecules in corresponding donor and acceptor channels are shown before (left panels) and 20 min after adding high salt (1 M NaCl) buffer (right panels). Scale bar, 5 μm . (C) Histograms of FRET efficiency as a function of time ($t = 0$ is when high salt was introduced) show the evolution of looped (high FRET) and unlooped (low FRET) populations. (D) Fraction of looped DNA (high FRET population) as a function of time, measured from the histograms in C. An exponential fit to this curve gives R . Error bars indicate $\pm$ SEM; $n = 5$.

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For example, for a 91-bp initial dsDNA with 10-nucleotide (nt) overhangs, looping was nearly irreversible and the looped high FRET state accumulated to saturation within about 20 min (Fig. 1, C and D). In this case, the looping rate

R could be determined by fitting the time evolution of the looped population with a single exponential function (Fig. 1D).

We measured R for a series of DNA molecules with a 10-nt overhang on each end and

with the circular size ranging from 67 to 106 bp (the circular size is the circumference of the DNA circle formed after looping and is therefore the sum of the initial dsDNA length and the overhang length). The measured looping time, $1/R$, varied from less than 10 min to more than 200 min (Fig. 2A, black squares). This 20-fold change in the looping times is smaller than expected; we expected that DNA much shorter than the persistent length would take considerably longer to form a loop. However, the result is qualitatively consistent with the observations that short DNA loops induced by the lac repressor and AraC protein can form efficiently in vivo and in vitro (2, 20–22), which suggests that the protein-induced looping may use the intrinsic flexibility of the DNA. Also, our result is consistent with a recent study which predicted that bending energy for short DNA loops would be independent of the loop length (13). In addition to the length dependence, a variation in looping rate depending on the angular phase between the two cohesive ends would be expected. Indeed, our data displays oscillation in R with a period of about one helical turn (see data points between 93 and 106 bp).

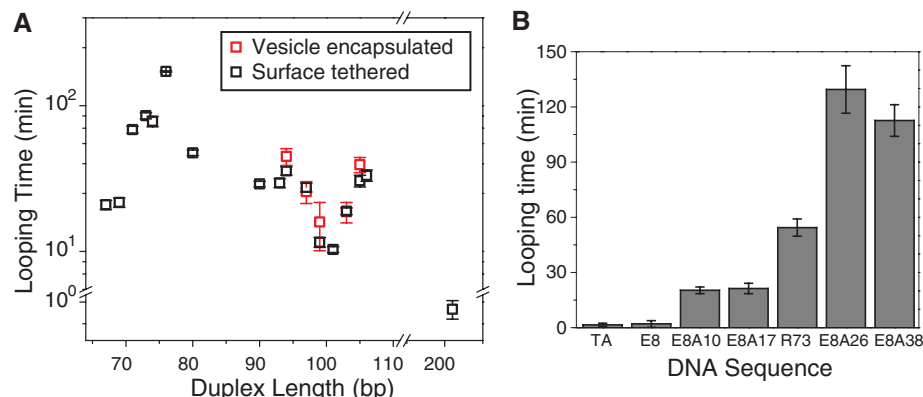


Fig. 2. (A) Looping time as a function of DNA circular length for surface-tethered DNA (black squares) and vesicle-encapsulated DNA molecules (red squares). (B) Looping time for 7 DNA sequences with 63-bp duplex length and 10-nt overhang. R73 is the standard sequence used in (A). Poly-A constructs were constructed by inserting $n = 10, 17, 26$, and 38 consecutive A bases in the middle of a random sequence (E8). Error bars indicate $\pm$ SEM; $n \geq 3$.

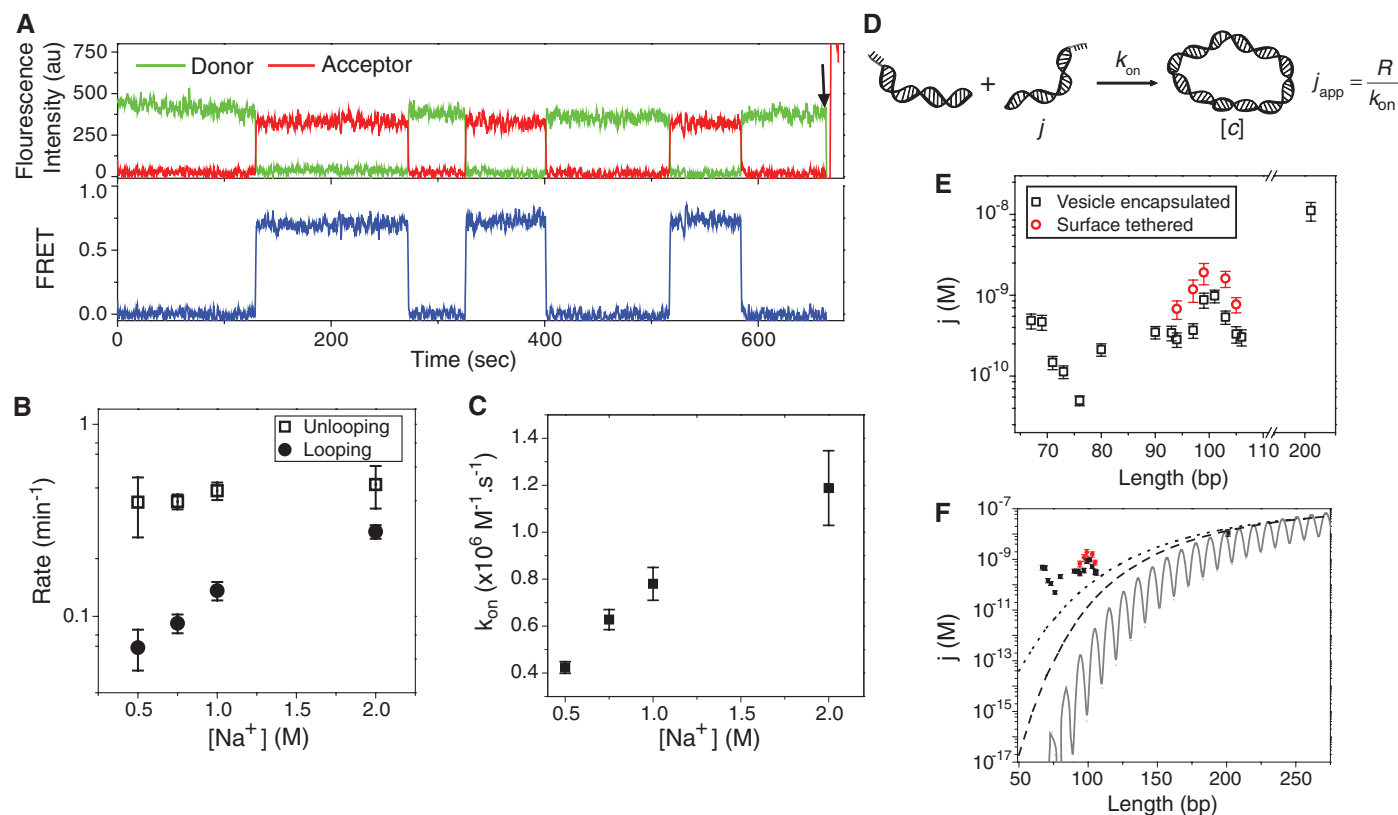
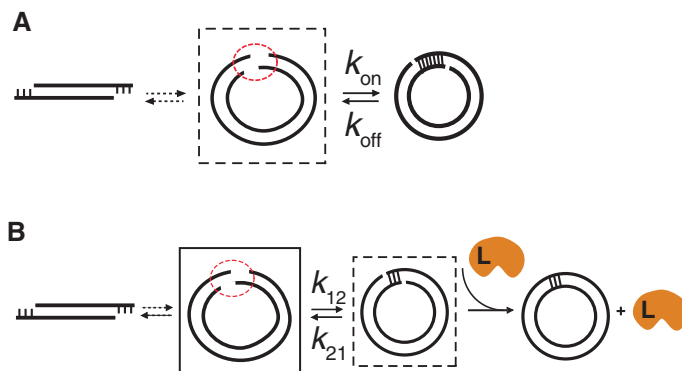


Fig. 3. (A) Representative fluorescence intensities (top, green for donor and red for acceptor) and corresponding FRET efficiency (bottom, blue) time traces measured from a single DNA molecule in 750 mM NaCl. The DNA has 91-bp initial dsDNA with 8-nt single-stranded overhangs. The arrow indicates a direct acceptor excitation to verify that the acceptor has not photobleached. (B) Looping and unlooping rates as a function of [NaCl]. The DNA has 91-bp initial dsDNA with 8-nt single-stranded overhangs. (C) k_{on} measured as shown in fig. S2 shows the same 3-fold increase as the looping rate with increasing

[NaCl]. Data are means $\pm$ SEM ($n \geq 300$ molecules). (D) The model to relate R , k_{on} , and apparent j factor. (E) j factor for surface-tethered DNA (black squares) and vesicle-encapsulated DNA (red circles). (F) Measured j factor for surface-tethered DNA (black squares) and vesicle-encapsulated DNA (red squares). Solid black curve is the Shimada-Yamakawa prediction for DNA cyclization. Dashed line and dotted line are the WLC predictions for the j factor of DNA circles with free boundary condition and for DNA molecules with 5-nm capture radius, respectively. Error bars indicate $\pm$ SEM; $n \geq 3$.

Fig. 4. (A) Our assay reports on the equilibrium population of the intermediate state (dashed box), with the two DNA ends in close proximity. **(B)** Schematic representation of DNA cyclization reaction steps in the ligase assay. The intermediate state with the two DNA ends in close proximity (solid box) does not get sampled in this assay. Instead, the ligase samples the equilibrium population of the annealed state (dashed box). Ligase protein is labeled L.



We performed a variety of controls to confirm that the unexpectedly weak length dependence of the looping rates was not an artifact of our experimental scheme. First, to rule out possible contributions from surface tethering and internal biotin labeling, we repeated the experiments for DNA molecules without any internal modification and confined in 200-nm-diameter phospholipid vesicles that are permeable to ions (23). Because infusion of 1 M NaCl ruptured the vesicles, we instead used 10 mM Mg^{2+} to stabilize the looped state. This is the same ionic condition used in the standard ligase assay. For five DNA constructs ranging from 94 to 105 bp, the looping times were similar between the vesicle encapsulated measurements and the surface measurements (Fig. 2A, red squares). Because DNA bending and torsional rigidity are sequence dependent (24), it remained possible that the high flexibility we observed was due to an extraordinarily flexible sequence. Therefore, we measured the looping times for a series of DNA constructs with identical loop length and overhang sequence but different internal base composition (Fig. 2B). We found that our standard sequence (R73) is not an outlier in terms of the looping rate. The DNA derived from a nucleosome positioning sequence (TA) (25) showed much faster (by a factor of 35) looping than our standard sequence R73 (7). We also examined the looping behavior of sequences with potentially curvature-inducing A tracks, $(A)_n$ —where $n = 0, 10, 17, 26$, or 38 consecutive A bases embedded in an otherwise randomly chosen sequence—and found that the looping rate varied from a factor of more than 30 higher ($n = 0$) to a factor of 2 lower ($n = 26$) than that of R73. The two orders of magnitude difference in the looping time for these sequences, despite the fact that the final 12 bp of these duplexes on both ends are similar, rules out duplex end opening as a possible mechanism for the rapid looping observed in our assay.

Changing the length of overhang to 8 nt allowed us to observe real-time looping-unlooping dynamics (Fig. 3A) and evaluate the rates directly as a functional of salt. Although the unlooping rate did not change between 0.5 M and 2 M Na^+ , the looping rate increased by a factor of 3 in this

range (Fig. 3B). However, because we observed the same increase in the bimolecular annealing rate (Fig. 3C), we can attribute the acceleration in looping at higher salt solely to the annealing enhancement. Therefore, monovalent ion concentrations above 0.5 M do not have a detectable effect on dsDNA flexibility (3).

We also investigated the effect of the single-stranded overhang length on the stability of DNA loops. Decreasing the overhang length from 10 nt to 9 or 8 nt while maintaining the initial duplex sharply increased the unlooping rate by about two orders of magnitude without an appreciable change in the looping rate (fig. S1A). Likewise, the equilibrium fraction of the looped state became progressively smaller by shortening the overhang (fig. S1B). Therefore, the main effect of longer overhangs in our assay compared to the 4-nt overhangs typical in the ligase assay is to increase the lifetime of the looped state. When a DNA loop forms, internal elastic energy stored in the loop is expected to provide a shear force that promotes unlooping. Indeed, we found that 8 bp of duplex melts 20 times as fast in a DNA circle as in a DNA dimer, likely due to internal tension in a circle that is absent in a dimer (see supplementary text and fig. S1C).

R can be calculated as the product of the bimolecular association rate k_{on} between 10-nt-long complementary strands and the effective concentration of one end of DNA in the vicinity of the other end, which we call the apparent j factor, j_{app} (5) (Fig. 3D). Using a similar surface-based assay but for intermolecular annealing (see supplementary text and fig. S2), k_{on} was measured to be $0.78 \pm 0.07 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ in 1 M NaCl and $0.26 \pm 0.04 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ in 10 mM Mg^{2+} , both consistent with an earlier estimate for short oligonucleotide annealing (26). The corresponding apparent j factors, calculated using $j_{\text{app}} = R/k_{\text{on}}$, are shown in Fig. 3E. Our calculated apparent j factors, along with the prediction of the WLC model (27, 28), are plotted in Fig. 3F. The solid line and the dashed line are the j factor for a semiflexible polymer with parallel and free boundary conditions, and the dotted line is the j factor for a polymer with free boundary condition and 5-nm capture radius (the two ends anneal when

they are closer than 5 nm) (29, 30). The measured j_{app} values matched the theoretical prediction for 201-bp DNA but deviated from the theoretical values by orders of magnitude for the shortest lengths examined, even under the most liberal boundary condition.

Our observation that the looping rate does not drop precipitously with decreasing DNA length is in stark contrast to the steep drop in the j factor predicted by the WLC model. In many biologically relevant protein-DNA interactions, such as in some genetic switches, the DNA bendability plays an important role in determining the state of the switch by controlling the concentration of one protein binding site in the vicinity of the other binding site (1, 31). Our assay samples such an equilibrium in which two DNA ends are in close proximity but not annealed (dashed box in Fig. 4A). In contrast, the ligase assay samples the equilibrium of the annealed state (dashed box in Fig. 4B) (5). The equilibrium looped population, which is the substrate for ligase protein, is very sensitive to the unlooping rate (figs. S1A and S1B). The looping rate is biologically more relevant because it reports on how quickly two regions of DNA are brought into close proximity, whereas the unlooping rate is additionally influenced by the melting rate of the short duplex formed. Our assay could independently measure the looping rate without being affected by the loop instability caused by internal tension in the short DNA circles. Many DNA binding proteins may have evolved to use the high flexibility of the DNA to capture and further stabilize transiently bent or looped DNA conformations.

Extended WLC models have previously been developed to explain the high flexibility of short DNA by allowing for the formation of temporary bubbles (32) or kinks (33), and molecular dynamics simulations observed the emergence of kinks in small DNA minicircles (34, 35). Others accommodate high bendability of short DNA by introducing nonharmonic elastic behavior (13). To gain insight into the mechanism of facile looping, we performed experiments on DNA constructs with a single backbone nick, double nicks, or a single-bp mismatch in the middle and observed one to two orders of magnitude higher looping rate compared with our original DNA constructs (figs. S3A and S3B). This considerable enhancement in the looping rate confirms that stable defects such as a single-bp mismatch or a nick can enhance global cyclizability of DNA, suggesting that similar but transient defects, if they are frequent enough, may explain the extreme bendability observed here. However, determining whether the high bendability of DNA at short length scales comes from transient kinks or bubbles or stems from anharmonic elasticity of DNA requires improved computational methods and further studies.

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Acknowledgments: We thank R. Phillips, H. Garcia, K. Ranganathan, R. Zhou, S. Doganay, A. Jain, K. Lee, G. Lee, and S. Arslan for helpful discussions. We are grateful to the late J. Widom for generous comments on an earlier version of this paper. This work was supported by U.S. National Science Foundation grants 0646550 and 0822613 and U.S. National Institutes of Health grant GM065367 to T.H. T.H. is an employee of the Howard Hughes Medical Institute.

Supplementary Materials

www.sciencemag.org/cgi/content/full/337/6098/1097/DC1
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1 May 2012; accepted 12 July 2012
10.1126/science.1224139

Network Context and Selection in the Evolution to Enzyme Specificity

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Enzymes are thought to have evolved highly specific catalytic activities from promiscuous ancestral proteins. By analyzing a genome-scale model of *Escherichia coli* metabolism, we found that 37% of its enzymes act on a variety of substrates and catalyze 65% of the known metabolic reactions. However, it is not apparent why these generalist enzymes remain. Here, we show that there are marked differences between generalist enzymes and specialist enzymes, known to catalyze a single chemical reaction on one particular substrate in vivo. Specialist enzymes (i) are frequently essential, (ii) maintain higher metabolic flux, and (iii) require more regulation of enzyme activity to control metabolic flux in dynamic environments than do generalist enzymes. Furthermore, these properties are conserved in Archaea and Eukarya. Thus, the metabolic network context and environmental conditions influence enzyme evolution toward high specificity.

Ancestral enzymes are proposed to have exhibited broad substrate specificity and low catalytic efficiency (*1*). Through mutation, duplication, and horizontal gene transfer, gene families diversified and promiscuous enzymes apparently were refined to exhibit specific and more efficient catalytic abilities (*2, 3*). Thus, today's metabolic enzymes are commonly assumed to be "specialists," having evolved to catalyze one reaction on a unique primary substrate

in an organism. However, some enzymes are "generalists" that promiscuously catalyze reactions on a variety of substrates in vivo (*2*) or exhibit multifunctionality by catalyzing multiple classes of reactions, often at different active sites (*4*). Thus, a fundamental question arises: Why do some enzymes evolve to become specialists, whereas others retain generalist characteristics? By analyzing enzyme functions and properties in experimental data and in silico biochemical network models, we show that the in vivo biochemical network context in which an enzyme resides may influence the evolution of enzyme specificity.

How many metabolic enzymes are generalists? To answer this question, we used a comprehensive reconstruction of the *Escherichia coli* K-12 MG1655 metabolic network, which accounts for the metabolic functions of 1260 gene products (28% of the predicted and experimentally validated open reading frames in *E. coli*) (*5*), which contribute to 1081 enzyme complexes analyzed in this study. In the reconstruction, we define a

reaction as a unique set of substrates that are chemically transformed into a unique set of products. With this definition, we classified 677 enzymes as specialists because they catalyze one unique reaction and 404 as generalists because they catalyze multiple reactions. Thus, we estimate that 37% of metabolic enzymes in *E. coli* are generalists, most of which exhibit substrate promiscuity (fig. S1A). Furthermore, specialist and generalist enzymes catalyze 454 and 859 metabolic reactions, respectively, distributed across many metabolic subsystems (Fig. 1, A and B). Thus, contrary to the textbook view of enzymes as "specific catalysts," generalist enzymes have a prominent role in *E. coli*, catalyzing at least 65% of the nonspontaneous metabolic reactions.

We performed several network-wide analyses to provide additional support for our estimates and the classification. First, we found that almost all genes in the network have been well characterized and studied in more than 61,727 published studies (fig. S1D). Second, we found no correlation between our classification and knowledge depth, i.e., neither specialist nor generalist enzymes had been studied in more depth (fig. S1E). Third, our generalist enzymes did not likely include many latent promiscuous reactions measured in vitro that likely do not occur in vivo, because 85% of the generalist enzymes reactions (GERxns) were active in silico in common growth conditions. This is the same percentage seen for specialist enzyme reactions (SERxns) (fig. S2). Fourth, because enzyme classification may vary with further study, we tested the sensitivity of the results presented in this work. We found the results to be qualitatively robust with improvements in the metabolic network from the discovery of new enzymes, variations in enzyme classification, and the exclusion of promiscuous enzymes or multifunctional enzymes from the generalist class (fig. S3). Although transporter reactions were not included in the groups of SERxns or

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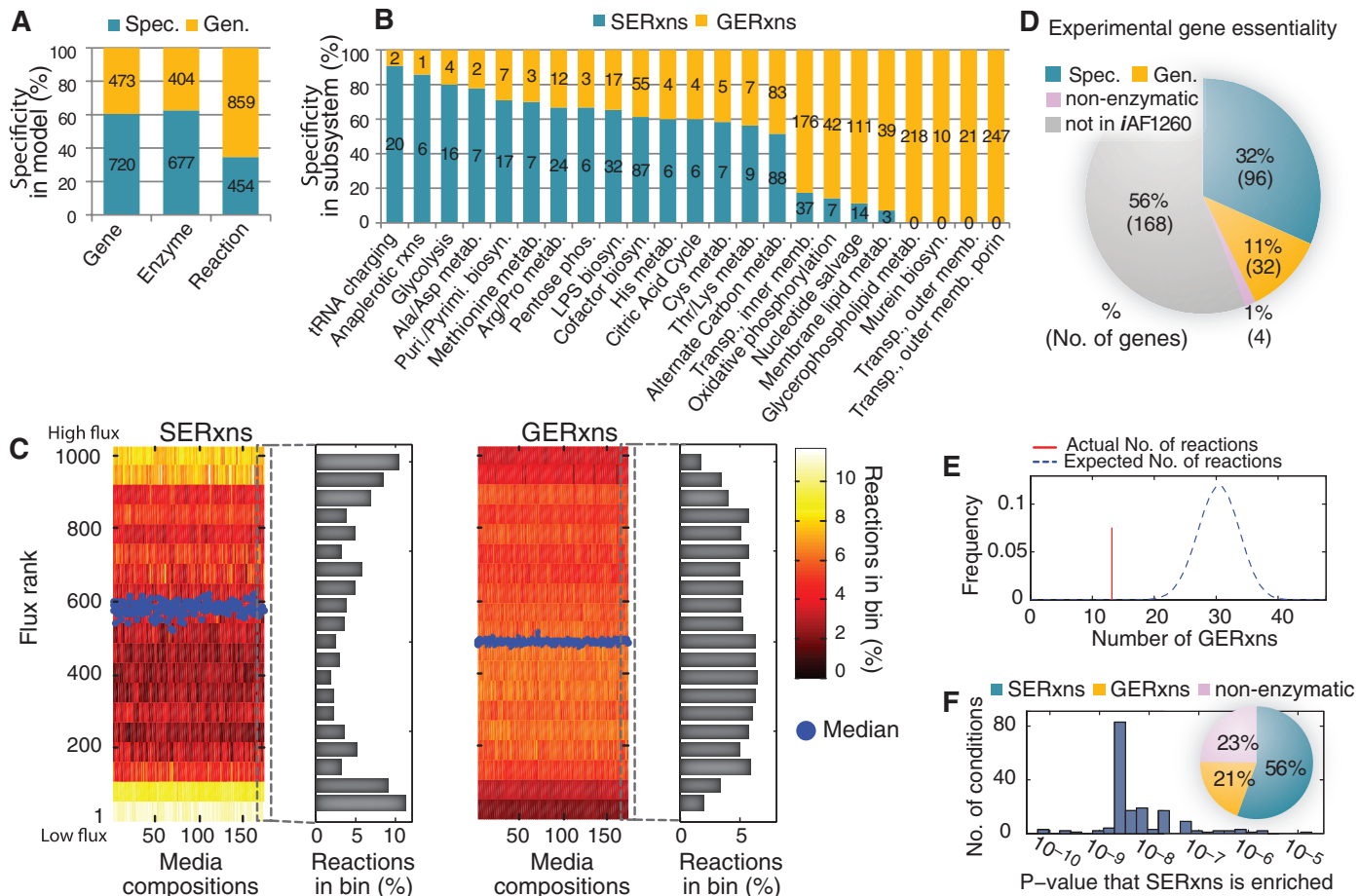


Fig. 1. (A) Specialist and generalist genes and proteins and their associated reactions were enumerated in *E. coli* metabolism. **(B)** Several metabolic subsystems were enriched in specialist enzyme reactions (SERxns) or generalist enzyme reactions (GERxns) in *E. coli* (hypergeometric $P \leq 0.05$). **(C)** Reaction flux magnitudes were rank-ordered and binned in histograms for each unique media condition. A heat map was used to visualize histograms for all 174 media conditions (columns) with each row representing bins spanning the given flux rank ranges (y axis). Color intensity shows histogram bin height, corresponding to the

percentage of reactions in the bin. Example histograms (shown on the right) provide for one representative condition. SERxns tend to have a higher flux, but low-flux SERxns are enriched in enzymes that synthesize low-abundance essential cell components, such as cofactors and prosthetic groups (fig. S4C). **(D)** Genes for specialist enzymes are more frequently essential in vivo. **(E)** In silico, few essential GERxns were identified for growth on glucose minimal medium. **(F)** For all 174 simulated growth conditions, SERxns are significantly enriched among in silico-predicted reactions essential for growth, representing 56% of the essential reactions (inset).

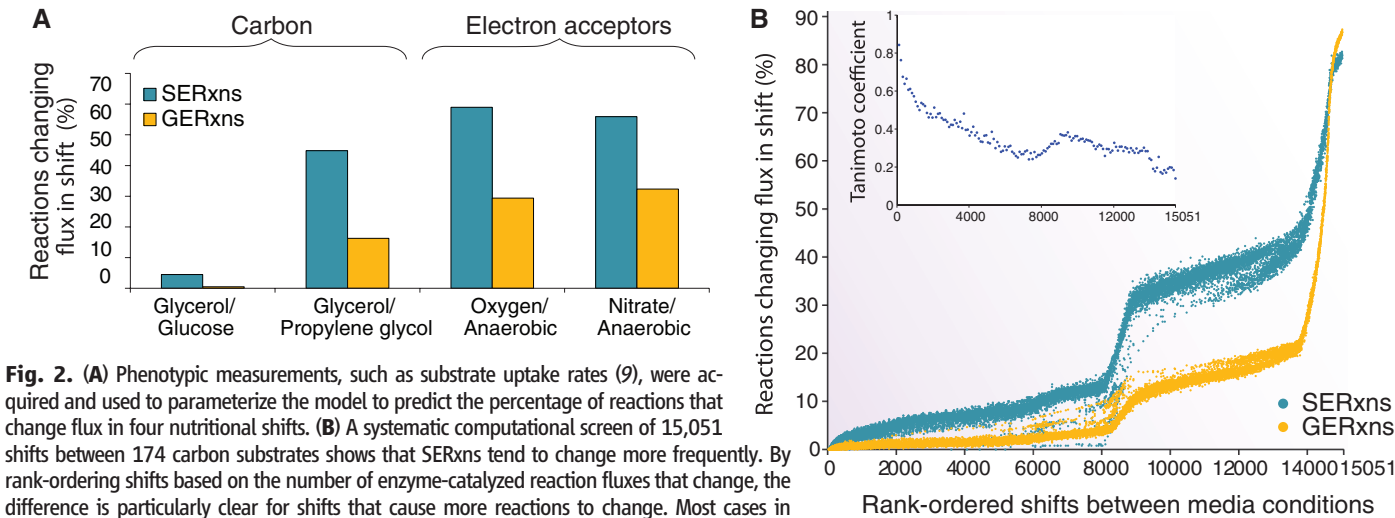


Fig. 2. (A) Phenotypic measurements, such as substrate uptake rates (9), were acquired and used to parameterize the model to predict the percentage of reactions that change flux in four nutritional shifts. **(B)** A systematic computational screen of 15,051 shifts between 174 carbon substrates shows that SERxns tend to change more frequently. By rank-ordering shifts based on the number of enzyme-catalyzed reaction fluxes that change, the difference is particularly clear for shifts that cause more reactions to change. Most cases in which there is only a weak difference involve shifts between two similar primary carbon substrates, as measured by their Tanimoto coefficients (inset; Tanimoto coefficients are averaged across sets of 100 shifts).

GERxns, their inclusion would not qualitatively change the results in this work (fig. S3). Thus, the classification and results from our subsequent analysis are robust.

Why are so many generalist enzymes evolutionarily retained, whereas others became specialists? Demands for higher metabolic flux may provide an evolutionary selective pressure to enhance an enzyme's catalytic rate and reduce the required enzyme concentration. However, catalytic improvements for one substrate of a generalist enzyme can suppress other catalytic activities (6). To determine if specialists maintain higher flux, we estimated the steady-state metabolic flux rates (7) for all *E. coli* enzymes using a genome-scale metabolic network model. We employed a Markov chain Monte Carlo sampling method (8) to simulate flux on 174 media conditions with different nutrient compositions (9). For each growth condition, the median flux for each reaction was rank-ordered to determine the relative flux among reactions.

Across all simulated growth conditions, SERxns maintained higher flux than GERxns (Fig. 1C and fig. S4). Gene duplications may have been fixed in the population when specialization occurred to increase activity of high-flux enzymes. Higher activity would permit lower enzyme concentrations, thereby offsetting the cost of duplication (10). Consistent with this reasoning, k_{cat} values are significantly higher for high-flux specialist enzymes than for all other enzymes (fig. S5C, Wilcoxon $P = 2.8 \times 10^{-7}$).

Although flux level may contribute to enzyme specialization, gene essentiality may also contribute. High substrate affinity for essential

enzymes could mitigate substrate competition in the synthesis of necessary biomass components, irrespective of flux level. Consistent with this hypothesis, we found that essential enzymes have lower K_m values and therefore higher substrate affinity (fig. S5F, Wilcoxon $P = 1.1 \times 10^{-11}$). Furthermore, specialist enzymes are enriched among experimentally determined essential genes (11) (hypergeometric $P = 8.65 \times 10^{-5}$, Fig. 1D). In silico simulation also demonstrated that cell growth rarely directly depends on flux through generalist enzymes (Fig. 1E), whereas many SERxns were essential for growth across all 174 tested media conditions (Fig. 1F and fig. S6).

Gene essentiality (12, 13) and reaction fluxes often vary (8, 14, 15) because natural environments are dynamic and nutrient concentrations fluctuate in the microbial microenvironment (16). The need to regulate reaction flux in dynamic environments could induce gene duplication and enzyme specialization to simplify the combinatorial complexity of regulating multiple reactions on a single enzyme (e.g., see serine hydroxymethyltransferase in fig. S7). To test this hypothesis, we identified enzymes that will require more metabolic regulation in dynamic environments by simulating changes in carbon source and electron acceptors for *E. coli*. For each substrate shift, the model predicted whether reaction flux should increase or decrease, and these predictions were consistent with measured differential gene expression (fig. S8) (17).

Across all shifts in growth media, there was a considerable difference in the percentages of active SERxns and GERxns that significantly changed their flux between growth conditions (Fig. 2A).

SERxns fluxes were often more than twice as likely to change than GERxns fluxes. Thus, flux through SERxns is considerably more sensitive to environmental change, whereas GERxns fluxes vary less. To examine if this is a general property, we simulated 15,051 pairwise environmental shifts. In 96% of these shifts, SERxns changed more frequently than GERxns (Fig. 2B). This difference was strongest for environmental shifts that cause more than 8% of the reactions to change flux (fig. S9). Because SERxns are subject to greater flux changes in nutritionally dynamic environments, it seems that duplication may have occurred to allow more focused regulation of fluxes. This duplication would be reinforced as the enzymes enhance their catalytic specificity.

In dynamic environments, metabolic flux can be regulated through metabolite-protein interactions or posttranslational modifications (PTMs) (18, 19). We quantified the association of metabolic regulation with enzyme specificity, using a few hundred metabolite-mediated regulatory interactions obtained from the EcoCyc database and enzyme PTMs from mass spectrometry studies in *E. coli* (9). Allosteric, uncompetitive, and noncompetitive regulatory interactions were enriched among specialists (hypergeometric $P = 9 \times 10^{-4}$), as were PTMs (hypergeometric $P = 5 \times 10^{-3}$). Metabolic regulation was less prevalent among generalists, consistent with the decreased need to change flux through their reactions in dynamic environments. Moreover, fluxes for reactions catalyzed by the same generalist often covary, thereby reducing requirements for more complex regulation (fig. S10).

To further assess the association of specificity with regulation, we quantified how frequently each reaction changed flux across all simulated 15,051 media shifts. K-means clustering elucidated three dominant reaction clusters (Fig. 3A). Two clusters show frequent changes in flux, and these were enriched in specialists, particularly those associated with central and amino acid metabolism (Fig. 3B). The reaction cluster with few changes in flux was significantly enriched in generalists (Fig. 3C). PTMs and small-molecule-mediated allosteric regulation were enriched within the cluster that experienced the most change in flux (hypergeometric $P = 5 \times 10^{-3}$), but depleted from the cluster dominated by generalists (hypergeometric $P = 3 \times 10^{-3}$; Fig. 3D and fig. S11). Thus, enzymes that exhibit more extensive metabolic regulation tend to have evolved increased enzyme specificity.

The aforementioned properties show how enzyme specificity correlates with holistic functions of the *E. coli* metabolic network. However, these properties should be conserved if they influence selection of enzyme specificity in protein evolution. Thus, we examined their conservation using genome-scale metabolic models of microbes from the other domains of life, including the archaeon *Methanosarcina barkeri* (20) and the eukaryotes *Saccharomyces cerevisiae* (21) and *Chlamydomonas reinhardtii* (22). Similar to *E. coli*, the three

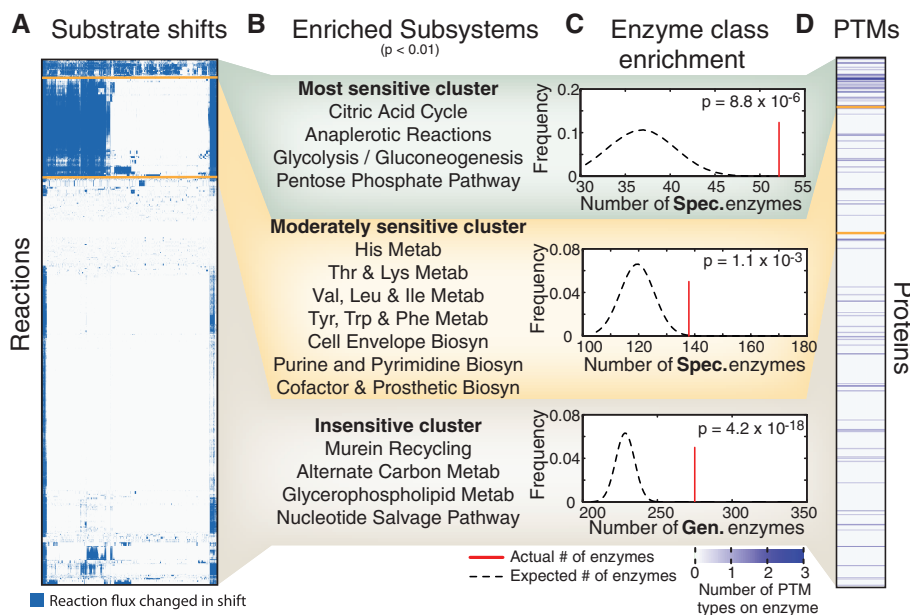


Fig. 3. (A) Clustering reactions that change (blue) or do not change (white) across 15,051 different media shifts (x axis) yields three distinct groups, (B) which are each enriched in unique metabolic subsystems. (C) Specialist enzymes are enriched in more sensitive clusters, whereas generalist enzymes are enriched in the cluster with few flux changes. (D) The number of PTMs (acetylation, phosphorylation, and/or succinylation) on enzymes increases with sensitivity of clusters.

organisms contain numerous generalist enzymes. Common growth conditions were simulated for each organism to estimate metabolic flux. In each organism, specialist enzymes maintained a higher flux on average than generalist enzymes. Moreover, when environmental shifts were simulated for each organism, generalist enzymes were less likely to change flux between growth conditions (fig. S12). Even as microbes diversified, high flux and a need for focused regulation in varying environments remained as general features of specialist enzymes.

It is generally believed that highly promiscuous ancestral enzymes eventually evolved to become specific and highly efficient (*1*). However, many current enzymes are only moderately efficient (*23*), and there are numerous generalists. Thus, evolution has not converged to a point where metabolic enzymes are all specialists. Our results suggest that this convergence has been hindered in part by the lower essentiality, smaller flux, and reduced regulatory requirements of generalist enzymes, including those that are multifunctional and those exhibiting substrate promiscuity (figs. S3B and S4C). The specialization of these enzymes may not provide adequate fitness advantages to offset the cost of gene duplication and maintenance (*10*) that accompanies the separation of catalytic functions into several specialists. In addition, these selective pressures may not influence some classes of enzymes if their generalist activities are desirable, such as in the degradation and clearance of diverse toxins (*24*) or the synthesis of structural lipids or glycoconjugates. However, our results suggest that many metabolic enzymes will specialize when an environmental change elicits a fitness challenge that

causes a generalist to contribute to the high-flux (*8*) or essential biomass-producing core (*25*) of metabolism, or if new environmental fluctuations require more focused regulation of flux. Preliminary analysis suggests that potential examples of this divergence include serine hydroxymethyltransferase and its isozyme LtaE (fig. S7) or pyruvate formate lyase and TdcE (see supplementary materials).

Our results demonstrate that the metabolic network, as a whole, supports organismal survival and influences cell physiology in a given environment. By analyzing the functions of its pathways and using biomolecular networks to integrate many disparate data types into a coherent whole, we show that systems biology allows the elucidation of selection pressures that are not apparent at the level of a single enzyme (*26–29*).

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Acknowledgments: We thank D. Zielinski for insightful discussion on this work. This work was supported by NIH, NSF, and U.S. Department of Energy grants 2R01GM057089-13, NSF GK-12 742551, DE-SC0004917, and DE-FG02-09ER25917. Data are available at the NCBI Gene Expression Omnibus (GEO) database (GSE34631).

Supplementary Materials

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21 November 2011; accepted 29 June 2012
10.1126/science.1216861

Synthesis of Methylphosphonic Acid by Marine Microbes: A Source for Methane in the Aerobic Ocean

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Relative to the atmosphere, much of the aerobic ocean is supersaturated with methane; however, the source of this important greenhouse gas remains enigmatic. Catabolism of methylphosphonic acid by phosphorus-starved marine microbes, with concomitant release of methane, has been suggested to explain this phenomenon, yet methylphosphonate is not a known natural product, nor has it been detected in natural systems. Further, its synthesis from known natural products would require unknown biochemistry. Here we show that the marine archaeon *Nitrosopumilus maritimus* encodes a pathway for methylphosphonate biosynthesis and that it produces cell-associated methylphosphonate esters. The abundance of a key gene in this pathway in metagenomic data sets suggests that methylphosphonate biosynthesis is relatively common in marine microbes, providing a plausible explanation for the methane paradox.

Methane plays a key role in the global carbon cycle and is a potent greenhouse gas. As such, knowledge of its

sources and sinks is essential to climate change models and to understand the flow of carbon within the biosphere. An unsolved problem in this

area is the observation that vast sections of the aerobic ocean are supersaturated with this gas, despite the fact that strictly anaerobic archaea are the only significant biological source of methane known (*1*). The amount of methane produced in these aerobic environments is substantial, constituting as much as 4% of the global methane budget (*2*). It has been suggested that anaerobic microenvironments within the aerobic ecosystem could allow the production of methane by known methanogens; however, this is contested on a variety of grounds [for a discussion, see (*1*, *3*)]. Recently, Karl *et al.* suggested a new model in which

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methane would be produced when aerobic marine microorganisms use methylphosphonic acid (MPn) as a source of phosphorus (2). The model is based on several observations: (i) a well-studied bacterial enzyme, carbon-phosphorus (C-P) lyase, produces methane from MPn (4); (ii), C-P lyase genes are abundant in marine microbes (5, 6); (iii) phosphonates make up a significant fraction of the available phosphorus pool in marine systems (7, 8); and (iv) the incubation of seawater microcosms with MPn leads to methane production (2). Although this model is conceptually appealing, it has an important missing link: MPn has never been detected in marine ecosystems, nor is it a known natural product. Moreover, based on known phosphonate biosynthetic pathways (9), it is difficult to see how MPn could be made without invoking unusual biochemistry.

With one exception, all known phosphonate biosynthetic pathways begin with formation of the C-P bond by the enzyme phosphoenolpyruvate mutase (Ppm) (9). We have used the *ppm* gene as a molecular marker to identify the genes responsible for synthesis of phosphonic acid antibiotics in numerous microorganisms (10–13). During the course of this work, we identified a putative phosphonate biosynthetic gene cluster in *Nitrosopumilus maritimus*, a member of the

ubiquitous group I marine Thaumarchaeota, whose members are among the most abundant organisms in marine surface waters (14, 15). Based on the experimentally validated functions of homologous enzymes (10, 16, 17), it is very likely that *N. maritimus* has the capacity to synthesize 2-hydroxyethylphosphonate (HEP), which is a common intermediate in phosphonate biosynthetic pathways (fig. S1A and table S1). Immediately adjacent to the putative HEP biosynthetic genes is an operon encoding a putative oxidoreductase, two putative sulfatases, and a protein of the cupin superfamily that we designated MpnS.

MpnS has weak homology to hydroxypropylphosphonate epoxidase (HppE) and hydroxyethylphosphonate dioxygenase (HepD), two enzymes that catalyze Fe(II)- and oxygen-dependent transformations of similar phosphonate substrates (figs. S1B and S2). Thus, we suspected that MpnS might be a similar phosphonate biosynthetic enzyme. To test this, we cloned and overexpressed the *mpnS* gene in *Escherichia coli* (18). Cell extracts containing MpnS stoichiometrically convert ^{13}C -labeled HEP to a product whose retention time and molecular mass are identical to those of MPn in liquid chromatography mass spectrometry (LC-MS) experiments (Fig. 1 and fig. S3). Using purified MpnS protein and HEP labeled with ^{13}C at either the 1- or 2- position,

we conclusively showed that the products of the MpnS reaction are MPn and HCO_3^- (Fig. 1, B and C). The MpnS-catalyzed reaction requires both Fe(II) and molecular oxygen but does not require an exogenous electron donor. Thus, like HepD, MpnS is an Fe(II)-dependent oxygenase that cleaves the unactivated C-C bond of HEP. However, the two enzymes catalyze distinct reactions. In the HepD reaction, the reducing equivalents needed for the incorporation of oxygen into the cleavage products are derived equally from the C-1 and C-2 carbons of HEP, whereas MpnS catalyzes the asymmetric oxidation of HEP, with all four electrons being derived from the C-2 carbon, affording the more reduced phosphonate product MPn.

Having shown that MpnS catalyzes the synthesis of MPn in vitro, using ^{31}P nuclear magnetic resonance (NMR) spectroscopy, we asked whether *N. maritimus* synthesizes phosphonic acids (Fig. 2A). The ^1H -decoupled ^{31}P spectrum of the soluble cell extract displayed two peaks in the 10- to 30-parts per million (ppm) range characteristic of phosphonic acids (19). The relative abundance of the two peaks varied with sample preparation and could be seen in both the soluble and cell debris fractions after sonication (fig. S4). Based on spiking of the sample with an authentic standard, neither peak can be

Fig. 1. In vitro assay of MpnS activity. **(A)** A crude cell extract from an *E. coli* MpnS overexpression strain was incubated aerobically with $1\text{-}^{13}\text{C}$ -HEP in the presence of Fe(II), and the phosphorus-containing products were examined with ^{31}P NMR spectroscopy. After incubation for 1 hour, a single product was observed as a doublet centered at 23.5 ppm. The mass and retention time of this product as determined by LC-MS are consistent with this product being $1\text{-}^{13}\text{C}$ -MPn (fig. S3). **(B)** After spiking of this reaction with the substrate, $1\text{-}^{13}\text{C}$ -HEP produced a second doublet centered at 19 ppm, showing that the substrate was completely consumed in the initial reaction. **(C)** The identity of the reaction products was determined with ^{13}C NMR after repeating the assay in a sealed vial, using purified MpnS with a mixture of $1\text{-}^{13}\text{C}$ -HEP and $2\text{-}^{13}\text{C}$ -HEP as substrates. The C-2-labeled carbon of HEP is converted to ^{13}C bicarbonate ($\text{H}^{13}\text{CO}_3^-$), whereas the C-1-labeled carbon is converted to $1\text{-}^{13}\text{C}$ -MPn. Bonding to phosphorus splits the ^{13}C peak in the NMR spectrum. Thus, the C-1 peak is split and the C-2 peak is not. Glycerol, a component of the assay mixture, is also observed in the ^{13}C spectrum. The ^{13}C label is indicated by an asterisk.

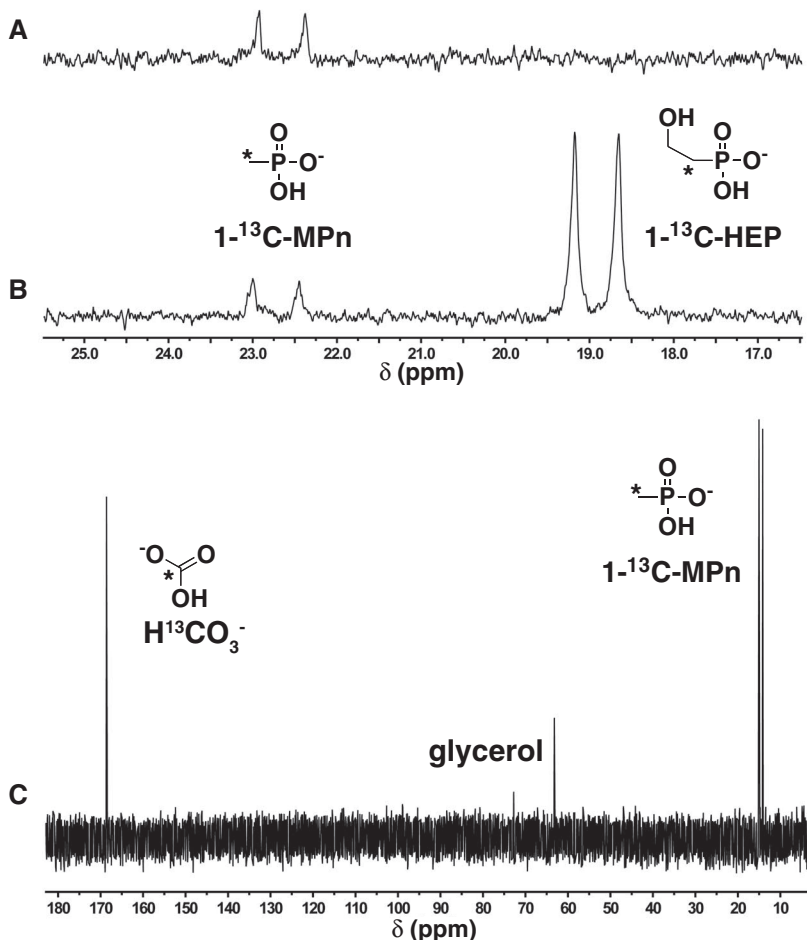
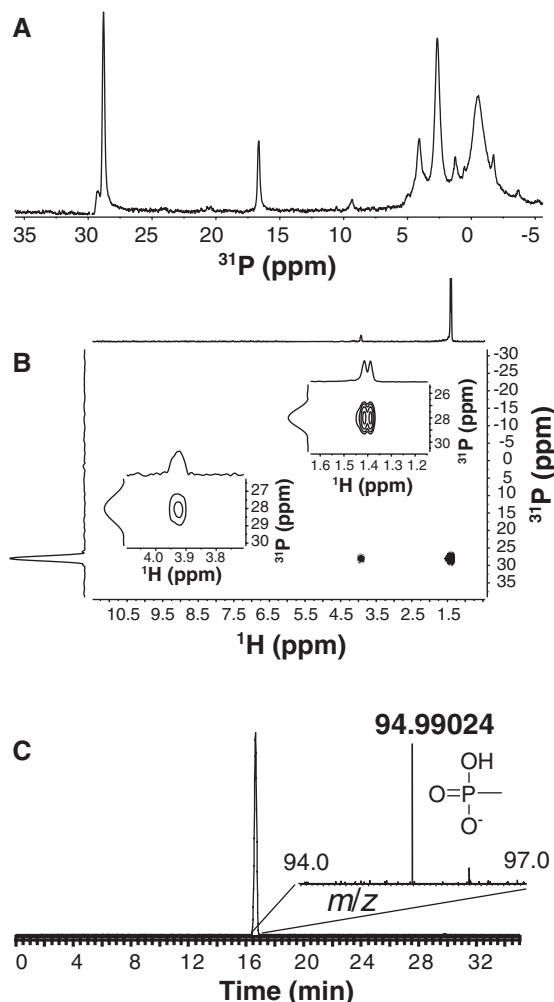


Fig. 2. In vivo production of methylphosphonate esters by *N. maritimus*. (A) A cell extract of *N. maritimus* was prepared by sonication of whole cells as described. After removal of the cell debris by centrifugation, the supernatant was examined by ^{31}P NMR spectroscopy, revealing at least two compounds with chemical shifts in the range typical of phosphonic acids. (B) The two-dimensional HMBC NMR spectrum of an *N. maritimus* cell extract. Comparison of the proton splitting patterns (shown in the insets) to those of model compounds (figs. S6 and S7) clearly shows that the P compound at 28 ppm in the ^{31}P dimension is a methylphosphonate ester. The doublet of the proton at 1.4 ppm coupled to the phosphorus is diagnostic for a methyl group bonded directly to phosphorus; that is, a methylphosphonate moiety. (C) High-resolution LC-MS analysis showing the presence of free methylphosphonate after strong acid hydrolysis of *N. maritimus* cell debris. The extracted ion chromatogram centered around a mass-to-charge ratio (m/z) of 94.99035 (the exact monoisotopic mass of methylphosphonate $[\text{M}-\text{H}]^-$) with a Fourier-transform mass spectrum and ion structure is shown in the inset. The chromatographic and MS fragmentation pattern is identical to that of an authentic MPn standard (fig. S8).



attributed to free methylphosphonate; however, because the phosphorus compounds are cell-associated, we expected them to be covalently linked to a larger, more complex molecule, thus changing the chemical shift in the ^{31}P NMR spectrum. Accordingly, we conducted a series of ^{31}P - ^1H heteronuclear multiple bond correlation (HMBC) experiments to identify the atoms linked to the P nuclei seen in the NMR spectra (Fig. 2B). Because the behavior of phosphonate esters in such experiments is not well documented, we also synthesized and characterized a series of phosphonate esters to support our assignments (figs. S5 to S7). Based on these experiments, the ^{31}P NMR peak at 28.7 ppm can be confidently assigned as an ester of methylphosphonate. Further support for this conclusion was provided by high-resolution MS, which revealed the presence of free methylphosphonate after strong acid hydrolysis of *N. maritimus* cell debris (Fig. 2C and fig. S8). Based on these results and the gene context of the MpnS locus (table S1), we suspect that *N. maritimus* synthesizes an exopolysaccharide decorated with methylphosphonate, similar to the HEP- and aminoethylphosphonate-modified polymers found in many bacteria and lower eukaryotes (20).

The data presented above suggest that *N. maritimus* produces a cell-associated methylphosphonate ester via an MpnS-dependent biosynthetic pathway. To link this finding to the larger marine environment, we screened the Global Oceanic Survey (GOS) metagenomic data set (21) for the presence of MpnS homologs. We also searched for homologs of the related HepD and HppE proteins. Initially, we screened the assembled

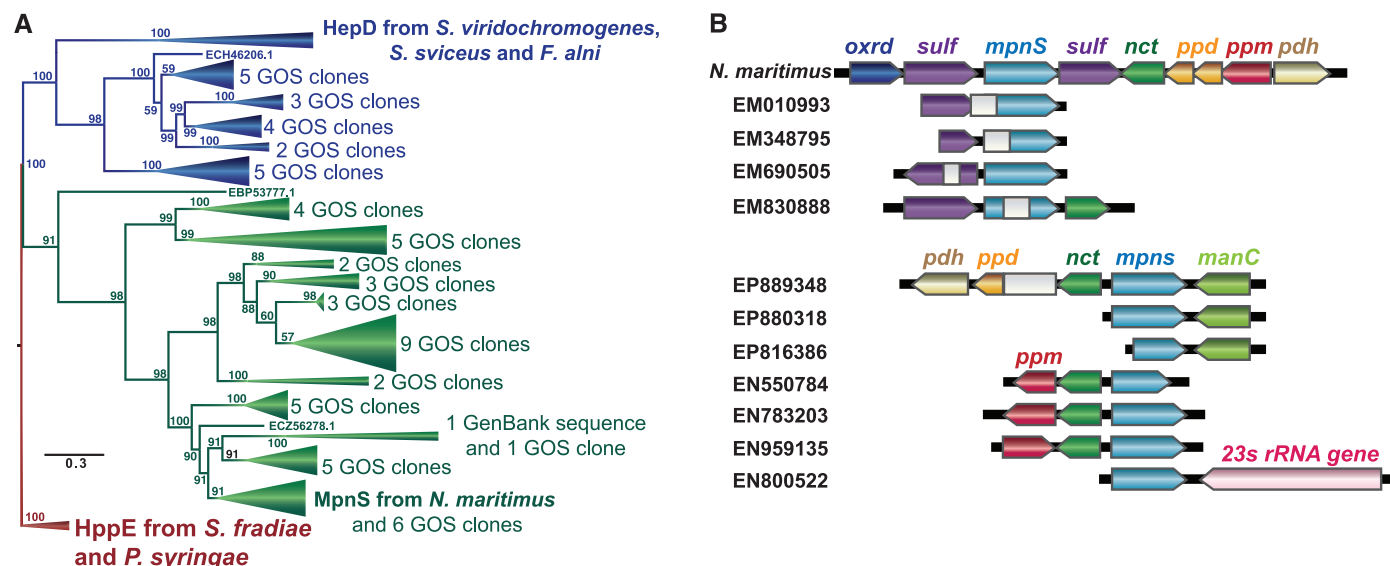


Fig. 3. (A) The evolutionary relationships of biochemically characterized MpnS, HepD, and HppE proteins (shown in bold) and homologs recovered from GenBank and the GOS metagenomic data set were inferred using maximum-likelihood analysis as described. Bootstrap values from 100 replicates are shown at the nodes. Robust bootstrap support for the tree shows that the method clearly differentiates MpnS

(green), HepD (blue), and HppE (red) proteins. The full tree with all individual homologs shown is presented in fig S9. (B) The gene content of large scaffolds containing the GOS MpnS homologs is compared to the *mpnS* locus of *N. maritimus*. The GenBank accession numbers for the GOS scaffolds are shown at left. The gray boxes represent sequencing gaps between paired-end reads.

GOS scaffolds, finding 46 MpnS and 20 HepD homologs, using a protein basic local alignment search tool (BLASTP) cutoff value of 10^{-10} (table S2). No HppE homologs were observed. None of the HepD homologs were identified when *N. maritimus* MpnS was used as the query sequence; likewise, none of the MpnS homologs were identified when HepD was used as a query. Thus, BLASTP clearly differentiates between the two homologous groups, supporting the assignment of the recovered sequences as MpnS and HepD proteins, respectively. To independently support these functional assignments, we constructed maximum-likelihood phylogenetic trees including biochemically validated MpnS, HepD, and HppE proteins (Fig. 3A and fig. S9). We also used a hierarchical clustering method to examine all putative and validated MpnS, HepD, and HppE proteins (fig. S10). In both cases, robust support for the functional assignments was obtained. Thus, we conclude that the recovered GOS MpnS homologs are likely to be methylphosphonate synthases.

Additional support for the function of the MpnS homologs was revealed by analysis of neighboring genes found in GOS DNA scaffolds (Fig. 3B and table S3). Many of the nearby open reading frames are homologous to those found in the *N. maritimus* gene cluster, including the phosphonate biosynthetic genes *ppm*, *ppd*, and *pdh*, as well as homologs of the sulfatases and nucleotidyl transferase genes, suggesting that the GOS scaffolds encode genes for the synthesis of similar MPn esters. Several other genes found on the scaffolds provide evidence for the identity of the organisms in which they are found. One of the scaffolds includes a 23S ribosomal RNA gene that can be confidently placed within the SAR11 clade between *Pelagibacter* species (fig. S11), whereas two of the *manC* genes are nearly identical to ones found in *Pelagibacter* sp. HTCC7211. Although the *mpnS* gene is absent in sequenced *Pelagibacter* genomes, these data strongly support the conclusion that some members of this genus have the capacity to synthesize MPn.

Relatives of *Nitrosopumilus* and *Pelagibacter* are among the most abundant organisms in the sea, with global populations estimated at 10^{28} for both ammonia-oxidizing Thaumarchaeota (14) and members of the SAR11 clade (22). Thus, the observation of *mpnS* in some members of these genera is consistent with the idea that MPn synthesis is prevalent in marine systems. To provide direct support for this notion, we measured the abundance of the *mpnS* gene relative to the abundance of typical single-copy genes as previously described (23). We also quantified the occurrence of the *ppm* gene to provide an estimate of the relative occurrence of phosphonate synthesis in general (table S4). Based on these data, we estimate that ~16% of marine microbes are capable of phosphonate biosynthesis, whereas 0.6% have the capacity to synthesize MPn. Because the GOS samples are confined to the upper few meters of the ocean, extrapolation of this anal-

ysis to the deeper ocean should be viewed with some skepticism. Nevertheless, the upper 200 m of the world's oceans are thought to contain $\sim 3.6 \times 10^{28}$ microbial cells, with an average generation time of ~2 weeks (24). Thus, even with the relatively modest abundance of MPn biosynthesis suggested by our data, it seems quite possible that these cells could provide sufficient amounts of MPn precursor to account for the observed methane production in the aerobic ocean via the C-P lyase-dependent scenario suggested by Karl *et al.* (2).

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Acknowledgments: This work was supported by the NIH (grants GM P01 GM077596 and F32 GM095024), the Howard Hughes Medical Institute (HHMI), and NSF (grants MCB-0604448, OCE-1046017, and MCB-0920741). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Institute of General Medical Sciences, NIH, NSF, or HHMI. The authors thank L. Zhu (University of Illinois at Urbana-Champaign) for valuable help with NMR experiments.

Supplementary Materials

www.sciencemag.org/cgi/content/full/337/6098/1104/DC1
Materials and Methods
Figs. S1 to S11
Tables S1 to S4
References (25–39)

31 January 2012; accepted 10 July 2012
10.1126/science.1219875

The Shared Antibiotic Resistome of Soil Bacteria and Human Pathogens

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Soil microbiota represent one of the ancient evolutionary origins of antibiotic resistance and have been proposed as a reservoir of resistance genes available for exchange with clinical pathogens. Using a high-throughput functional metagenomic approach in conjunction with a pipeline for the de novo assembly of short-read sequence data from functional selections (termed PARFuMS), we provide evidence for recent exchange of antibiotic resistance genes between environmental bacteria and clinical pathogens. We describe multidrug-resistant soil bacteria containing resistance cassettes against five classes of antibiotics (β -lactams, aminoglycosides, amphenicols, sulfonamides, and tetracyclines) that have perfect nucleotide identity to genes from diverse human pathogens. This identity encompasses noncoding regions as well as multiple mobilization sequences, offering not only evidence of lateral exchange but also a mechanism by which antibiotic resistance disseminates.

The continued evolution and widespread dissemination of antibiotic resistance genes in human pathogens is a preeminent clinical challenge (1). Environmental reservoirs have long been implicated as a source of resistance found in human pathogens (2). However, apart from certain opportunistic bacterial pathogens, among which the same species can be found in the environment or infecting humans (3), examples of resistance genes from environmental bacteria with high identity to those of pathogens

are rare (4, 5). The two documented examples are of *Kluyvera* and *Shewanella* isolates, which are found free-living in environmental settings (5, 6) yet have resistance genes (CTX-M β -lactamase and *qnrA* genes, respectively) with high identity (100% identity in clinical *Kluyvera* isolates) to those of pathogens (4, 5). The limited examples of resistance genes shared between environmental microbes and human pathogens raise questions regarding the clinical impact of environmental resistance. For instance, whether shared resistance

is confined to genes of particular mechanisms (such as enzymatic β -lactam cleavage) or applies to many genes with diverse mechanisms of resistance is unknown. Additionally, whether a single horizontal gene transfer (HGT) event between environment and clinic can result in the de novo acquisition of a multidrug-resistant phenotype is unclear. The two previous reports of high-identity resistance genes shared between environmental and pathogenic bacteria did not find evidence of colocalized resistance genes or of syntenic mobilization elements (4, 5), hallmarks of transferable multidrug resistance (7, 8). Determining the clinical impact of environmental resistance requires a deeper profiling of environmental reservoirs for the organisms and genotypes most likely to exchange resistance with human pathogens.

Soil, one of the largest and most diverse microbial habitats on earth, is increasingly recognized as a vast repository of antibiotic resistance genes (9–13). Not only does soil come into direct contact with antibiotics used extensively in rearing livestock (14) and plant agriculture (15), but it is also a natural habitat for the Actinomycete genus *Streptomyces*, whose species account for the majority of all naturally produced antibiotics (16). Despite numerous studies demonstrating that soil contains resistance genes with biochemical mechanisms similar to those in common pathogens (3, 11–13), the sequence identities of these genes diverge from those of pathogens (17), providing little evidence that these resistomes have more than an evolutionary relationship. Therefore, whether soil has recently contributed to or acquired resistance genes from the pathogenic resistome remains an open question, and accordingly, the role of soil in the current global exchange of antibiotic resistance remains poorly defined.

To examine the capacity of nonpathogenic, soil-dwelling organisms to exchange antibiotic resistance with human pathogens, we sought to select for organisms prone to this exchange. Because many major clinical pathogens are Proteobacterial (18), we cultured multidrug-resistant Proteobacteria from the soil (19), with the aim of enriching for resistance genes shared between soil and human pathogens. We interrogated the resistome of the resulting culture collection using functional metagenomic selections, which are ideally suited to characterize acquirable resistance because they identify any gene sufficient to confer resistance to a new host (such as a path-

ogen) (20). To facilitate the rapid and efficient functional characterization of metagenomic libraries, we developed a massively parallel, multiplexed functional selection platform that enables simultaneous sequencing, de novo assembly, and functional annotation of hundreds of resistance fragments from many independent selections (termed PARFuMS: Parallel Annotation and Re-assembly of Functional Metagenomic Selections) (fig. S1) (19).

We applied PARFuMS to a collection of 95 soil-derived cultures (“AB95”), representing bacteria with high-level resistance to various antibiotics. Cultures were obtained from 11 U.S. soils (table S1), passaged serially through minimal and rich media containing one of 18 antibiotics at 1000 mg/L (tables S2 and S3) (21), and subjected to 16S ribosomal DNA (rDNA) profiling (19). We confirmed that the culture collection was enriched for Proteobacteria and dominated by traditional soil-dwelling organisms (such as *Pseudomonas* and *Pandoraea*) (fig. S2). Equal proportions of the 95 cultures were pooled, and bulk genomic DNA was extracted. One- to 3-kb fragments of this metagenomic DNA were cloned into an expression vector and transformed into *Escherichia coli*. The resulting 2.57-Gb metagenomic library was selected on solid culture medium containing 1 of 12 antibiotics representing amino acid derivatives, aminoglycosides, amphenicols, β -lactams, and tetracyclines, at concentrations to which the host-strain was susceptible (table S4). Resistance was detected against all 12 antibiotics, and resistance-conferring fragments were sequenced, assembled, and annotated by using PARFuMS, yielding 161 contigs (N50 >

1.7 kb). Of the 252 open reading frames (ORFs) identified, 110 (44%) could confidently be annotated as antibiotic resistance genes (by similarity to a known resistance gene, which was consistent with functional selection), whereas another 62 (25%) were categorized as resistance-related (Fig. 1, A to C, and table S5).

Of the 110 resistance genes, 18 had 100% amino acid identity to entries in GenBank, and another 32 were highly similar ($\geq 90\%$ identity). Thus, although we recovered several genes previously identified, most of the resistance genes discovered (54%) were formerly unknown (Fig. 1D). For instance, we identified a gene conferring D-cycloserine resistance from an AB95 isolate (most closely related to *Serratia ficaria*) for which sequence alone could not predict resistance function (19). The ORF was 92% identical to a protein of unknown function from *Serratia proteamaculans* 568 (CP000826) (Fig. 2A) and enabled *E. coli* to tolerate high concentrations of D-cycloserine (128 $\mu\text{g/mL}$) (Fig. 2B). The D-cycloserine resistance protein had low-level identity to a drug/metabolite transporter (46% identity over 91% of the sequence; YP_001583420), indicating that the gene may have efflux-related function, which is consistent with known mechanisms of D-cycloserine tolerance (22).

Of the 110 AB95 resistance genes, 55 were β -lactamases. The majority of these sequences clustered with class C β -lactamases and were dissimilar to entries currently in GenBank (fig. S3), which is a common result from metagenomic experiments (11, 20, 23). AB95 β -lactamases were highly divergent from those of the antibiotic-

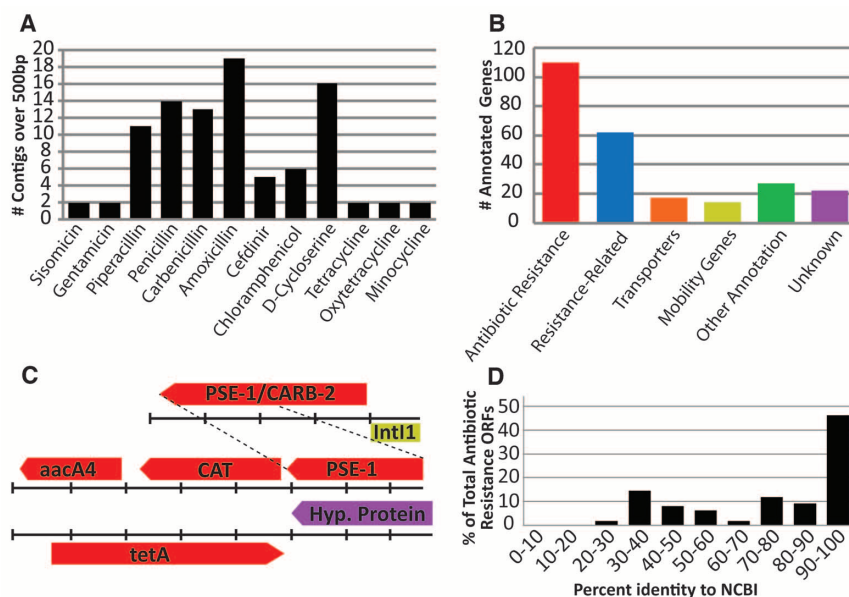


Fig. 1. Functional selection of the AB95 soil metagenomic library with 12 antibiotics (19). (A) Bar chart depicting the number of distinct contigs over 500 base pairs (bp) recovered from selection with each of the 12 antibiotics. (B) Functional classification of ORFs predicted by PARFuMS, across all selections. (C) Three representative metagenomic fragments; colors match categorizations depicted in (B). The distance between tick marks is 300 bp, and dashed lines indicate common sequence on two distinct fragments. (D) Amino acid identity between antibiotic-resistance ORFs and the closest hit from GenBank, across all selections.

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producing *Streptomyces*, indicating ancient evolutionary relationships (fig. S3 and table S6). However, several β -lactamases with >99% identity to sequences from both soil and enteric organisms were recovered (fig. S3).

We identified 16 sequences, from 10 selections, with 100% nucleotide identity to antibiotic resistance genes previously sequenced from clinical isolates of many common human pathogens (Table 1). A bacterium was considered pathogenic only if it was isolated from an infection in a diseased human host. The 16 sequences represent seven different genes, conferring resistance to five classes of antibiotics (β -lactams, aminoglycosides, amphenicols, sulfonamides, and tetracyclines) (Table 1). We discovered multiple examples of syntenic, soil-derived resistance genes shared with many common pathogens. For example, a chloramphenicol-acetyltransferase with 99.7% identity to *K. pneumoniae* clinical isolates was adjacent to both an aminoglycoside-acetyltransferase and a β -lactamase identical to genes found in many pathogens (JX009248). Additionally, an insert from two selections contained *aadB* (an aminoglycoside-adenyltransferase) ad-

jacent to *qacEΔ1* (an efflux pump conferring antiseptic resistance) and *sulI* (a dihydropteroate synthase conferring sulfonamide resistance) in a class 1 integron-like structure (JX009286). All three genes and much of the surrounding integron (>2 kb) are 100% identical to numerous clinical pathogens. The seven soil-derived resistance genes (Table 1) are globally distributed amongst human pathogens: Clinical isolates from many countries and all major continents contain genes with perfect nucleotide identity to genes from this set (fig. S4).

To identify soil isolates from the AB95 culture collection harboring the aforementioned resistance genes, we performed polymerase chain reactions using primers specific to the boundaries of the predicted ORFs (19). We identified two organisms isolated from farmland soil containing six of the resistance genes identical to pathogens, as well as two additional genes with over 99% identity to those in pathogens (tables S7 and S8) (19). We confirmed that seven genes were present in an organism most closely related to *Pseudomonas* sp. K94.23 [a member of the *P. fluorescens* complex (24)], three originated

from a strain most similar to *Ochrobactrum anthropi*, and two were in both genomes (19). *P. fluorescens* is not believed to cause human infection (25), and there are only limited examples of *O. anthropi* subgroups known to infect humans (26). Rather, these two organisms are predominantly found in environmental settings (25, 27). The substantial phylogenetic divergence between these traditionally nonpathogenic soil isolates and numerous human pathogens (table S9) contrasts with the 100% identity of numerous resistance genes found in both groups, confirming that these genes moved between species via HGT.

Three ORFs from *O. anthropi* and *P. fluorescens*, conferring β -lactam, aminoglycoside, and amphenicol resistance and representing one gene shared by both organisms and one specific to each, were cloned from their genomic DNA, expressed in *E. coli*, and verified for resistance to seven antibiotics (19). In all cases, the ORFs conferred resistance at concentrations 16-fold greater than that of an empty-vector control and enabled growth in a minimum of 128 μ g/mL (and up to 2048 μ g/mL) of antibiotic (Table 2). These results mirror the minimum inhibitory concentrations of the source soil strains (Table 2), demonstrating that the resistance genes retain functionality even when removed from all native genomic context, emphasizing their broad host-range compatibility.

Perfect nucleotide identity between full-length resistance genes from distinct species implies that recent HGT has occurred between these organisms (28)—evidence that has not been previously reported between a nonpathogenic soil-dwelling organism and human pathogens. The seven resistance genes we discovered encompass all major mechanistic classes of antibiotic resistance (29) and are identical to genes found in diverse human pathogens, representing both Gram-negative and -positive bacteria. Moreover, for five of the soil-derived contigs that share resistance genes with pathogens, at least 80% of the contig is identical to sequence from a clinical isolate, encompassing coding and noncoding regions alike (the maximum span of identity is 2.28 kb) (table S10). In support of recent mobilization, we found 11 distinct sequences annotated as either an integrase or transposase from six antibiotic selections. Two *intI1* integrases were adjacent to resistance genes from both our organisms and pathogens, indicating a shared mechanism of HGT between soil and pathogenic bacteria. Four of the contigs assembled from our set are over 99% identical to a large span of sequence, found in numerous pathogens, that contains a high density of resistance genes and is flanked by multiple mobility elements (Fig. 3). This cluster of resistance genes exhibits extensive modularity; many combinations of the individual resistance elements are present in a multitude of clinical pathogens.

The closest homologs to each AB95 resistance gene include pathogenic resistance genes that are chromosomal as well as plasmid-borne, implying a diverse genetic organization of these

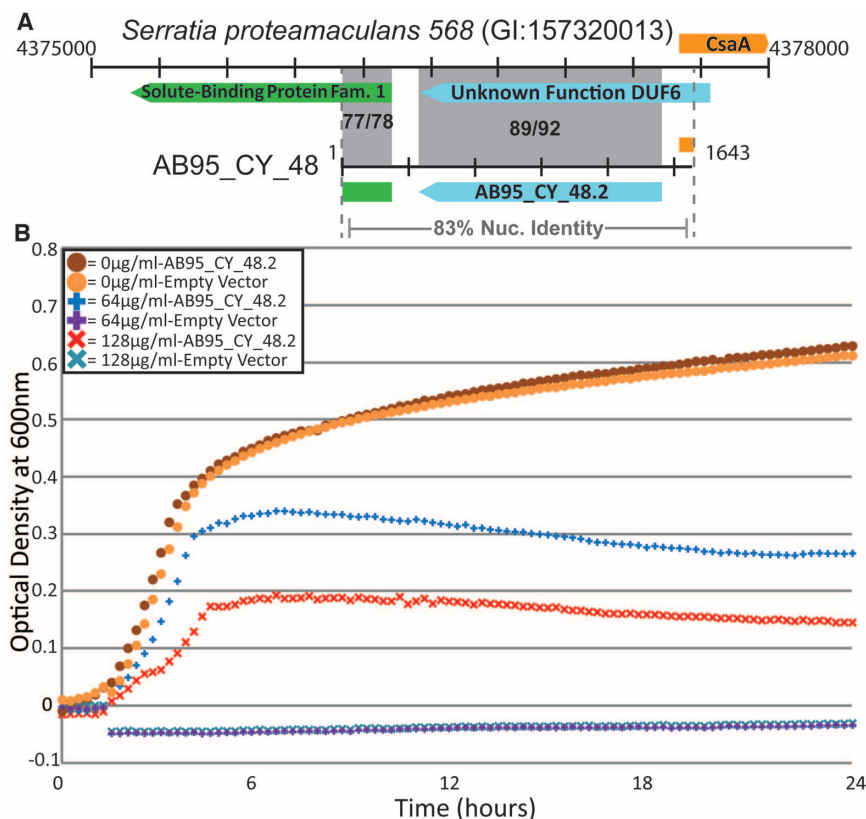


Fig. 2. A gene conferring resistance to D-cycloserine was captured for which sequence was unable to predict resistance function. (A) Resistance-conferring fragment AB95_CY_48 compared with its closest hit from the National Center for Biotechnology Information (NCBI) nucleotide collection. ORFs of the same color indicate homologous sequence; both nucleotide and amino acid percent identities are given in shaded regions (nucleotide/amino acid). Base-pair coordinates flank sequences, and the distance between each tick mark is 300 bp. (B) Measurements of absorbance at 600 nm, taken every 15 min, depict growth of *E. coli*, containing either AB95_CY_48.2 or an empty vector at clinically relevant concentrations of D-cycloserine. Measurements are corrected for background absorbance from media-only controls and are averages of three trials (19).

implicating conjugation or transformation as potential mechanisms of HGT (table S11). Additionally, we discovered nine integrases/transposases proximal to resistance genes not yet identified in

Given the extensive interspecific transfer of antibiotic resistance, and our data suggesting recent exchange between soil bacteria and clinical pathogens, we sought to identify routes of dissemination between these reservoirs. Possibilities include direct exchange between soil microbes and human pathogens or indirect transfer via reservoirs such as the human intestinal microbiota. Many resistance genes from the intestinal microbiota are identical to those found in diverse human pathogens (20), and accordingly, we compared the AB95 resistance genes with a set of resistance genes from cultured intestinal isolates (20), a collection of 128 representative gut organisms (table S12), and resistance genes from fecal metagenomes (19, 20). Most AB95 resistance genes were dissimilar to sequences from any intestinal data set, with the average amino acid identity ranging from 30.2 to 45.5% (fig. S5). However, the two cultured data sets contained perfect matches to distinct AB95 resistance genes (table S13). One such AB95 gene (JX009365) was not only identical to *tetA* from an intestinal isolate, but also to numerous pathogens, including *A. baumannii*, *E. coli*, *K. pneumoniae*, and *S. typhimurium*, indicating potential interconnections between the resistomes of the human gastrointestinal tract, soil, and clinical pathogens.

The exchange of resistance between soil and pathogens emphasizes the clinical importance of the soil resistome, regardless of whether resistance genes are moving from soil to the clinic, or vice versa. Transmission from soil to clinic establishes soil as a direct source of pathogenic resistance genes. Movement of resistance from pathogens into soil means pathogens can transfer resistance

Gene name	GenBank ID	Number of selections*	Antibiotic class	Annotation [mechanism]	Pathogens hit (GI number)
AB95_PI_68.1	JX009363	4	β-lactam	blaP1 [enzymatic degradation]	<i>A. baumannii</i> (94960156), <i>K. pneumoniae</i> (114147191), <i>P. aeruginosa</i> (117321883), <i>S. typhimurium</i> (12719011), <i>P. mirabilis</i> (157674381)†
AB95_CH_13.1	JX009364	1	Amphenicol	Chloramphenicol efflux [efflux]	<i>A. baumannii</i> (169147133), <i>P. aeruginosa</i> (260677483)
AB95_TE_2.2	JX009366	3	Tetracycline	tetA(G) [efflux]	<i>A. baumannii</i> (169147133), <i>S. typhimurium</i> (12719011)
AB95_TE_1.1	JX009365	3	Tetracycline	tetA [efflux]	<i>A. baumannii</i> (169147133), <i>E. coli</i> (312949035), <i>K. pneumoniae</i> (290792160), <i>S. typhimurium</i> (37962716)†
AB95_GE_3.3	JX009367 JX009373	2	Aminoglycoside	aadB [covalent modification]	<i>E. cloacae</i> (71361871), <i>K. pneumoniae</i> (206731403), <i>P. aeruginosa</i> (37955767), <i>S. typhimurium</i> (17383994)†
AB95_GE_3.1	JX009368 JX009374	2	Sulfonamide	sul1 [target modification]	<i>C. diphtheriae</i> (323714042), <i>E. cloacae</i> (71361871), <i>K. pneumoniae</i> (206731403), <i>P. aeruginosa</i> (37955767), <i>S. typhimurium</i> (17383994), <i>Yersinia pestis</i> (165913934)†
AB95_CH_21.1	JX009369	1	Aminoglycoside	aacA4 [covalent modification]	<i>A. baumannii</i> (164449567), <i>K. pneumoniae</i> (238865601), <i>P. aeruginosa</i> (219872982), <i>S. typhi</i> (34014739)†

*Number of selections in which the entirety of a given gene was captured. †More pathogens exist for which 100% nucleotide identity was observed than listed

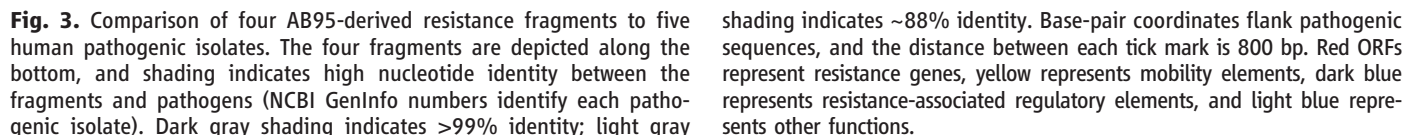


Table 2. Minimum inhibitory concentrations of various antibiotics toward both multidrug resistant soil isolates and *E. coli* clones expressing selected resistance genes (all concentrations are $\mu\text{g/mL}$). AX, amoxicillin; CA, carbenicillin; PE, penicillin; PI, piperacillin; CF, cefdinir; CH, chloramphenicol; SI, sisomicin; GE, gentamicin; MN, minocycline; OX, oxytetracycline; TE, tetracycline; and blank cells indicate inhibitory concentrations were not determined.

	AX	CA	PE	PI	CF	CH	SI	GE	MN	OX	TE
<i>Ochrobactrum</i> soil isolate	>2048	>2048	>2048	>2048	<16	512	512	512	<4	256	64
<i>Pseudomonas</i> soil isolate	>2048	>2048	>2048	>2048	>1024	1024	>1024	>1024	8	128	32
AB95_PI_68.1	>2048	>2048	2048	2048							
AB95_CH_33.1						256					
AB95_GE_3.3							>1024	>1024			
<i>E. coli</i> + empty vector control	<16	<32	64	16	<8	<8	<8	<8	<8	8	4

to soil organisms, of which many can cause nosocomial infection and may emerge as pathogens, akin to the rise of *A. baumannii*.

Powered by PARFuMS, a method for characterizing functional selections at <1% of the cost of traditional approaches (19), we describe antibiotic resistance genes found in nonpathogenic soil-dwelling bacteria and of all major mechanistic classes (29) with perfect nucleotide identity to many diverse human pathogens. We also show that multiple resistance genes are colocalized within long stretches of perfect nucleotide identity and are flanked by mobile DNA elements. These findings not only provide evidence for recent HGT of multidrug resistance cassettes between soil and clinic, but also a mechanism through which this exchange may have occurred.

The *Ochrobactrum* and *Pseudomonas* isolates originated from farmland soils fertilized with manure from antibiotic-treated livestock. However, our current study design did not enable a statistically significant association of pathogen-identical resistance genes to specific soils. Rather, our results highlight the fact that soil and pathogenic resistomes are not distinct, emphasizing the clinical importance of environmental resistance. Our new method provides the increased throughput required to power future studies to identify soil (11), aquatic (5), and other (20) environments prone to resistance exchange with human pathogens and to understand how specific anthropogenic practices influence the likelihood of this dissemination (3, 23).

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Acknowledgments: We thank R. Mitra for initial discussions regarding simulations of metagenomic assembly; T. Druley for discussions surrounding the use of the six β -lactamase control fragments; J. Fay for discussions on dating horizontal gene transfer; J. Gordon for support, thoughtful discussion, and as the advisor to A.R.; A. Moore for naming PARFuMS; and the Genome Technology Access Center at Washington University in St. Louis for generating Illumina sequence data. This work was supported by awards to G.D. through the Children's Discovery Institute (award MD-II-2011-117), the International Center for Advanced Renewable Energy and Sustainability at Washington University, and the National Academies Keck Futures Initiatives, Synthetic Biology-SB2. M.O.A.S. received funding from the Lundbeck foundation and the European Union FP7-HEALTH-2011-single-stage grant agreement 282004, EvoTAR. K.J.F. is a NSF graduate research fellow (award DGE-1143954). A.R. is the recipient of an International Fulbright Science and Technology Award. The data reported in this paper are described in the supplementary materials. Raw sequencing reads have been deposited to MG-RAST with accession nos. 4489630-39, 4489641-43, 4489645-46, 4489648-49, 4489650-51, 4489653-57, 4489659, 4489661-63, 4489665, and 4489667-68. Assembled sequences have been deposited to GenBank with accession nos. JX009202 to JX009380. The authors declare no competing financial interests.

Supplementary Materials

www.sciencemag.org/cgi/content/full/337/6098/1107/DC1
Materials and Methods
Figs. S1 to S7
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References (30–39)

20 February 2012; accepted 22 June 2012
10.1126/science.1220761

TLR13 Recognizes Bacterial 23S rRNA Devoid of Erythromycin Resistance-Forming Modification

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Host protection from infection relies on the recognition of pathogens by innate pattern-recognition receptors such as Toll-like receptors (TLRs). Here, we show that the orphan receptor TLR13 in mice recognizes a conserved 23S ribosomal RNA (rRNA) sequence that is the binding site of macrolide, lincosamide, and streptogramin group (MLS) antibiotics (including erythromycin) in bacteria. Notably, 23S rRNA from clinical isolates of erythromycin-resistant *Staphylococcus aureus* and synthetic oligoribonucleotides carrying methylated adenosine or a guanosine mimicking a MLS resistance-causing modification failed to stimulate TLR13. Thus, our results reveal both a natural TLR13 ligand and specific mechanisms of antibiotic resistance as potent bacterial immune evasion strategy, avoiding recognition via TLR13.

Toll-like receptor 2 (TLR2), TLR4, and TLR9 are major host sensors of Gram-negative bacteria, and TLR2 is thought to be the central detector of Gram-positive bacteria,

whereas other pattern-recognition receptors (PRRs) such as TLR7 contribute to bacteria sensing as well (1–7). However, the high sensitivity of mice lacking expression of these TLRs to Gram-positive

bacteria implies that other TLRs or members of other classes of PRRs—such as C-type lectins, RIG-I-like helicases (RLHs), or nucleotide binding domain- and leucine-rich repeat-containing receptors [NOD-like receptors (NLRs)]—play a role in the detection of Gram-positive bacteria. We therefore compared the responsiveness of macrophages lacking the expression of molecules that signal downstream of these PRRs, including caspase recruitment domain (CARD) 9, receptor-interacting protein 2, apoptosis-associated speck-like protein containing a CARD, interleukin-1 (IL-1) receptor, IL-18, or MyD88, to heat inactivated *Staphylococcus aureus* (hiSa) or *Streptococcus pneumoniae* (both Gram-positive) in the presence of a TLR2-blocking antibody (see supplementary materials and methods section) (2, 8–10). We

found that cytokine production strictly depends on MyD88 (fig. S1A), which suggests that TLRs rather than RLHs or NLRs are responsible for the detection of these bacteria. Moreover, analysis of ectopically expressed RLH function indicated a lack of RLH involvement in Gram-positive bacteria sensing (fig. S1B).

Next, we asked whether endosomal TLRs (TLR3, -7, -8, -9, -11, and -13) are involved in cell activation. We inhibited endosomal acidification with bafilomycin and analyzed UNC93B1-mutant (3D) macrophages that lack endoplasmic reticulum–endosome TLR trafficking and are susceptible to *S. aureus* infection (2, 11, 12). Bafilomycin treatment abrogated recognition of Gram-positive bacteria in *Tlr2*^{+/+} macrophages (Fig. 1A). Furthermore, 3D/*Tlr2*^{+/+} and 3D/*Tlr2*^{−/−} mice or corresponding macrophages (but not those generated from 3D mice unless TLR2 was blocked) were unresponsive to a Gram-positive bacterial challenge (Fig. 1, B and C, and fig. S1C). Unexpectedly, *Tlr23479*^{−/−} macrophages (or mice) responded well to a hiSa challenge, unless the bacterial preparations were subjected to ribonuclease A (RNase A) treatment, which did not impair TLR2-driven activation of wild-type (WT) controls, or endosomal TLR function was abrogated (Fig. 1, D to F). These data suggested that an endosomal RNA sensor besides TLR3 and TLR7 can act as cellular detector of hiSa.

Dendritic cell (DC) subsets express different sets of TLRs (13). We generated bone marrow–

derived conventional (c) DCs and plasmacytoid (p) DCs in vitro. The responsiveness of these cells to hiSa was dependent on MyD88 and UNC93B1. Specifically, *Tlr23479*^{−/−} CD8^{high} (expressing TLR11, TLR12, and TLR13) and signal regulatory protein α (Sirp)^{high} cDCs (expressing TLR13 but lacking TLR11 and TLR12) responded to hiSa, whereas *Tlr23479*^{−/−} pDCs (expressing TLR12 but lacking TLR11 and TLR13) failed to do so (Fig. 1G). Together, these findings imply that TLR13 acts as a bacterial single-stranded (ss) RNA sensor, even though TLR13 has recently been linked with the recognition of vesicular stomatitis virus (14).

To identify the relevant RNA, we incubated hiSa with calf intestinal phosphatase, 5'-phosphate-specific phosphatase [to affect the integrity of 16S and 23S ribosomal RNA (rRNA)], or double-stranded RNA-specific RNase III or VI. These treatments did not alter the stimulatory activity of hiSa, in line with a recent report (fig. S2, A to C) (15). However, ssRNA-specific RNase A treatment abrogated the *Tlr23479*^{−/−} cDC (and macrophage) stimulatory activity of hiSa, as did nucleic acid-degrading benzonase [Fig. 1, D, E, and G, and fig. S2B; note that Flt3L-expanded CD8⁺ cDCs do not produce IL-12p70 in response to TLR2 ligands that are contained in hiSa (16)]. We then treated total RNA with 5'-phosphate-dependent exonuclease (to degrade specifically large rRNAs, namely 16S and 23S rRNA) and purified large rRNAs (fig. S2C) to narrow down the

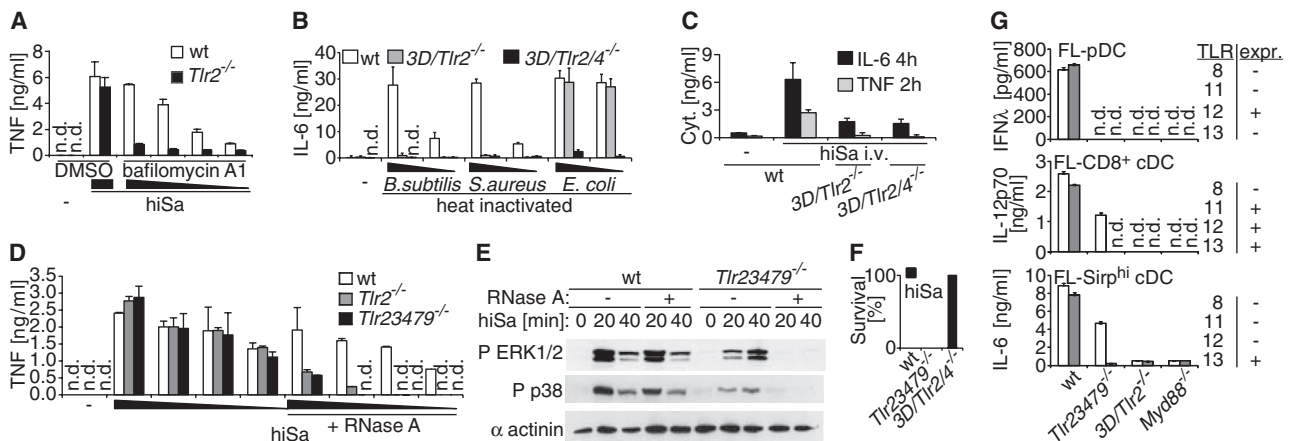


Fig. 1. Gram-positive bacteria and their RNA activate *Tlr23479*^{−/−} macrophages and DCs via an unknown TLR. (A) Macrophages were preincubated for 30 min with dimethyl sulfoxide (DMSO) alone or 50 nM bafilomycin A1 and were challenged for 8 hours with 10^9 colony-forming units (CFU)/ml heat-inactivated *S. aureus* (hiSa; DMSO) or 10^9 , 10^8 , 10^7 , and 10^6 CFU/ml hiSa (bafilomycin A1; −, unchallenged). Supernatants were analyzed by enzyme-linked immunosorbent assay (ELISA). n.d., not detected. (B) Macrophages were challenged for 16 hours with 10^9 or 10^8 CFU/ml of bacterial suspensions, whereas (C) corresponding mice were challenged intravenously (i.v.) with 10^9 CFU hiSa or PBS only (−) at 2 and 4 hours, upon which serum was drawn. Supernatants and serum samples were analyzed by ELISA. Cyt., cytokine; TNF, tumor necrosis factor. One out of three experiments with similar results and, respectively, $n = 3$ mice per group is illustrated as mean $\pm$ SD (error bars). (D and E) Macrophages were challenged for 16 hours (D) or for the times indicated (E) with untreated (−) or RNase A-treated (+) hiSa suspensions. (D) 10^9 , 10^8 , 10^7 , and 10^6 or (E)

10^8 CFU/ml hiSa was used for the challenge, upon which supernatants were analyzed by ELISA or lysates were analyzed by SDS–polyacrylamide gel electrophoresis and immunoblotting. P, phosphorylated; ERK, extracellular signal-regulated kinase. (F) Mice were challenged by injection with hiSa (1.6×10^{11} CFU/kg body weight) and α -D-galactosamine (800 mg/kg body weight) intraperitoneally 45 min after intravenous injection of IFN- γ (50 μ g/kg body weight). Survival was monitored, and all deaths occurred within 16 hours of treatment ($n = 6$ deaths per WT and 3D/*Tlr2*^{+/+} groups, $n = 4$ for *Tlr23479*^{−/−} mice). (G) Flt3L-antigen-derived DC subsets were challenged with untreated (white bars) or RNase A-treated (gray bars) hiSa at 5×10^6 CFU/ml for 16 hours. Supernatants were analyzed for cytokine contents by bead array. The respective TLR expression (expr.) in DC subsets is indicated (−, not detectable expression; +, expression). (A to E and G) For each panel, representative results from at least three experiments are shown, and each illustrated data point (A to D and G) represents mean $\pm$ SD (error bars) of duplicates.

stimulatory activity. After transfection, large rRNA isolates of both *S. aureus* and *Escherichia coli* triggered the activation of *Tlr23479*^{-/-} macrophages and cDCs, whereas 16S/23S rRNA digestion abrogated stimulatory activity (Fig. 2A). Accordingly, low-molecular weight portions from total RNA lacked stimulatory activity, whereas high-molecular weight portions of Gram-positive and Gram-negative bacterial RNA activated *Tlr23479*^{-/-} cells (Fig. 2B and fig. S2, D and E). These findings suggested that a fraction of large bacterial rRNAs activates macrophages and cDCs in a MyD88-dependent manner. We assume that the increased RNA-driven activation of *Tlr23479*^{-/-} macrophages in comparison to WT cells reflects a lack of TLRs competing for downstream signal transduction molecules.

To analyze whether rRNA modifications induced in antibiotic-resistant strains by antibiotic treatment [e.g., with erythromycin (17, 18)] would modify the immunostimulatory capacity of rRNA, we applied five clinical *S. aureus* isolates displaying various resistance phenotypes, including erythromycin resistance. Isolates grown in the presence of erythromycin largely lacked the capacity to activate *Tlr23479*^{-/-} macrophages and induced lower amounts of serum cytokines early after infection (2 hours) of *Tlr23479*^{-/-} mice (Fig. 2, C and D). In contrast, WT as well as *Tlr23479*^{-/-} mice and corresponding macrophages responded largely normally toward the same isolate grown in the absence of erythromycin (Fig. 2, C and D, and fig. S2, F to H). The later (16 hours) increase and equalization of serum cytokine levels independent of erythromycin treatment (fig. S2H) suggested the loss of 23S rRNA methylation in the absence of erythromycin within the host. Together, these results demonstrate an

erythromycin-driven camouflage of RNA from its receptor. Specifically, N6 methylation of rRNA adenosine (A) 2085 in *S. aureus* (corresponding to *E. coli* A2058) by the erythromycin resistance methyltransferase B (ermB) or ermC confers macrolide, lincosamide, and streptogramin group (MLS) antibiotic (including erythromycin) resistance (17, 18). Accordingly and also in line with the inducibility of erm expression by erythromycin (17, 18), 23S rRNA from *S. aureus* grown in erythromycin failed to stimulate *Tlr23479*^{-/-} macrophages (Fig. 2E). In contrast, 23S rRNA from resistant *S. aureus* not grown in erythromycin and 23S rRNA from *E. coli* (including enterohemorrhagic *E. coli*) activated *Tlr23479*^{-/-} macrophages, whereas the respective 16S rRNAs failed to do so (Fig. 2E and fig. S2, I and J). Moreover, overexpression of ermB and ermC (the latter being subcloned from cDNA of an erythromycin-grown *S. aureus* isolate) in *E. coli* and *Bacillus subtilis* strains not only conferred erythromycin resistance but also ablated 23S rRNA stimulatory activity (Fig. 2F and fig. S2K). These data indicate that resistance to MLS group antibiotics (including erythromycin) mediated by site-specific methylation (targeting A2085 in *S. aureus* and A2058 in *E. coli* 23S rRNA) rendered 23S rRNA nonstimulatory.

To address the immune stimulatory activity of 23S rRNA in more detail, we designed three oligonucleotides (ORNs) as analogs of *S. aureus* 23S rRNA segments, each of which carries an A in its center that becomes methylated constitutively or under growth restriction to modulate the docking of protein synthesis cofactors or antibiotics. The three ORNs named SaI, SaII, and SaIII represented *S. aureus* A1662 [*E. coli* A1616, methylation of which promotes fitness (19)],

S. aureus A2530 [*E. coli* A2503, targeted by chloramphenicol, florfenicol, and clindamycin resistance RNA methyltransferase (20)], and *S. aureus* A2085 [*E. coli* A2058, modification of which costs fitness (17, 18, 21)], respectively (table S1).

Only SaIII (which mirrors *S. aureus* A2085) activated *Tlr23479*^{-/-} cells (Fig. 3A). pDCs recognized SaIII via TLR7, but this activity was lost with 3'-terminal deletion (fig. S3). ORNs resulting from deletions of 3'- and 5'-termini (SaIIId3, SaIIId5, Sa23) equally activated *Tlr23479*^{-/-} cDCs (Fig. 3B), whereas preincubation of *S. aureus* RNA or of ORN Sa23 with an antisense SaIII RNA strand (SaIIIs) abrogated the stimulatory activity (Fig. 3C). These results indicated single-strand structure and singularity of the stimulatory activity within the bacterial transcriptome. Successive terminal deletions toward a 12-mer ORN (Sa12, table S1) led to sequences that were identical in *S. aureus* and *E. coli* 23S rRNAs. Length-dependent reduction of stimulatory capacity could largely be compensated by terminal fill-ups (Sa12A19, Fig. 3D) (22). Upon N6 methylation at A6 (corresponding to *S. aureus* A2085 and mimicking erm-methylated 23S rRNA), Sa12 lacked stimulatory capacity, whereas N6 methylation at A7 merely caused a partial reduction (Fig. 3E). Consecutive single substitutions of Sa12 revealed "CGGAAAGACC" as the minimal stimulatory segment because ORNs with substitutions at position one or two of Sa12 (Sa12s1 and Sa12s2) were fully stimulatory, whereas further substitutions resulted in drastic loss (Sa12s10 and Sa12s12) or abrogation of the stimulatory activity (Fig. 3F and table S1).

In contrast, Sa12 derivatives mimicking eukaryotic 28S rRNA or specific 23S rRNA mutations that render bacteria resistant to MLS antibiotics

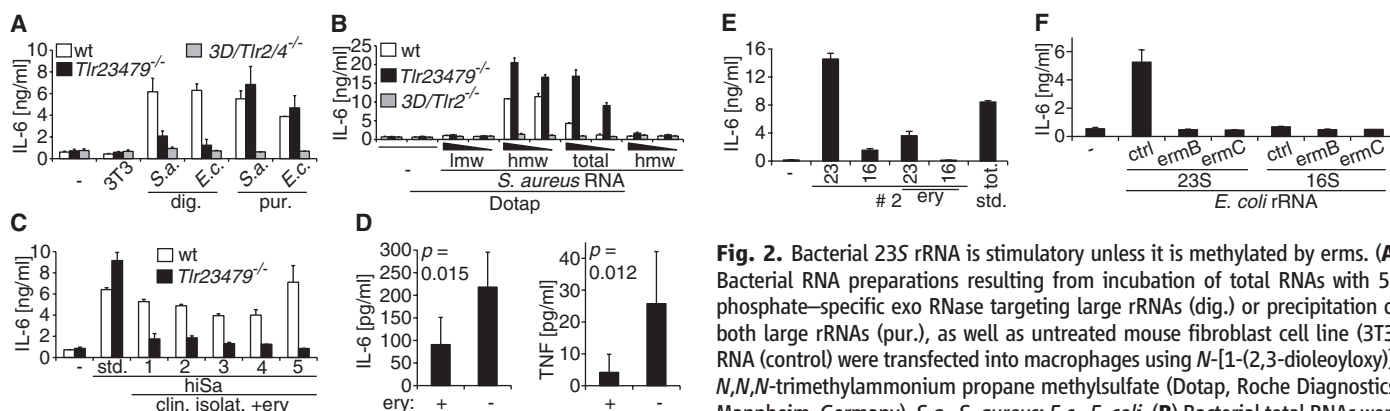


Fig. 2. Bacterial 23S rRNA is stimulatory unless it is methylated by erms. (A) Bacterial RNA preparations resulting from incubation of total RNAs with 5'-phosphate-specific exo RNase targeting large rRNAs (dig.) or precipitation of both large rRNAs (pur.), as well as untreated mouse fibroblast cell line (3T3) RNA (control) were transfected into macrophages using *N*-[1-(2,3-dioleoyloxy)]-*N,N,N*-trimethylammonium propane methylsulfate (Dotap, Roche Diagnostics, Mannheim, Germany). *S.a.*, *S. aureus*; *E.c.*, *E. coli*. (B) Bacterial total RNAs were separated by anion-exchange chromatography into low-molecular weight (lmw) and high-molecular weight (hmw) fractions, which were used to challenge macrophages with or without Dotap transfection. (C) Macrophages were challenged with 10^9 CFU/ml erythromycin-sensitive (std.) *hiSa* or five erythromycin-resistant clinical *S. aureus* isolates cultured in 10 mg/liter erythromycin (clin. isolat. +ery). (A to C) Supernatants were analyzed 16 hours poststimulation using ELISA. (D) *Tlr23479*^{-/-} mice were infected i.v. with 10^8 CFU erythromycin-resistant *S. aureus* clinical isolate growing logarithmically in the presence (+) or absence (-) of erythromycin. Serum was drawn after 2 hours and analyzed for cytokines by cytometric bead array. Mean $\pm$ SD (error bars) for $n = 6$ mice per each group is shown. (E) Total (tot.) RNA from erythromycin-sensitive *S. aureus* (std.) and agarose gel-purified 16S (16) and 23S (23) rRNAs from clinical isolate 2 grown in the absence or presence of erythromycin (ery) were transfected into *Tlr23479*^{-/-} macrophages using Lyovec (Cayla-InvivoGen, Toulouse, France). (F) *E. coli* BL21 was transformed with empty vector control (ctrl) or ermB or ermC expression plasmids. After 16 hours, culture of 16S and 23S rRNA was isolated and transfected into *Tlr23479*^{-/-} macrophages. (E and F) Supernatants were analyzed 16 hours postchallenge using ELISA. (A to C, E, and F) Each panel illustrates a representative result of three independent experiments and depicts means $\pm$ SD (error bars) of duplicate samples. For (D), one statistically significant experiment has been performed ($P \leq 0.015$).

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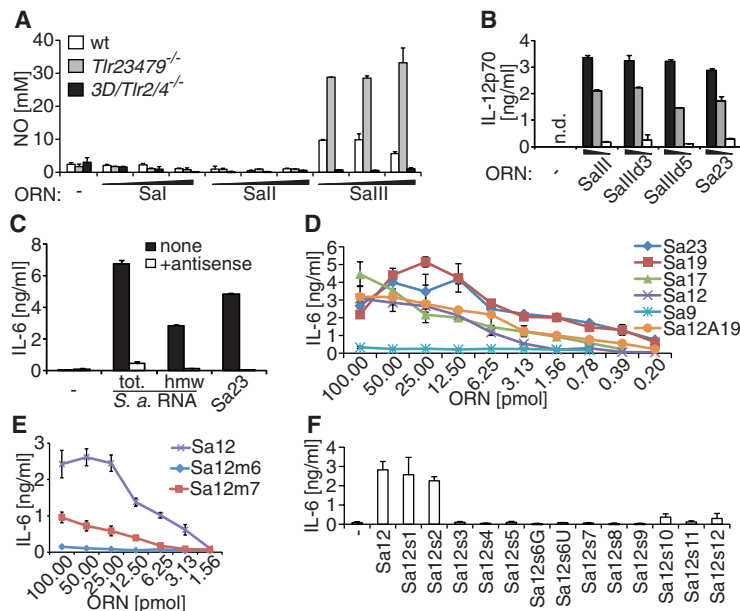
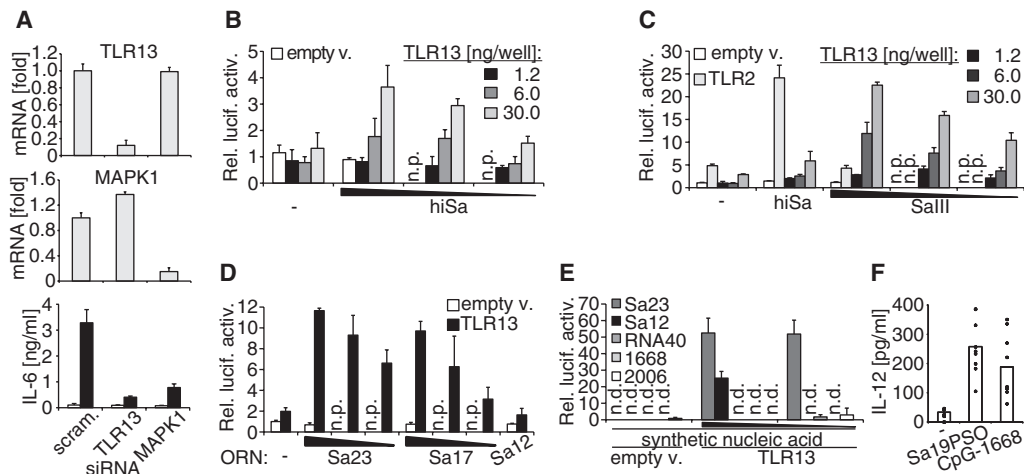


Fig. 3. Oligoribonucleotides (ORNs) covering the erm target site in 23S rRNA (region around A2085 in *S. aureus*/2058 in *E. coli*) activate macrophages and cDCs. (A) Sequence motifs covering three separate methylation sites in *S. aureus* 23S rRNA were mirrored by ORNs (see table S1). Macrophages were challenged with 1, 10, and 100 pmol per well of the ORNs. NO, nitrite. (B) *Tlr23479*^{-/-} FL-CD8⁺ cDCs were transfected with the ORNs indicated (amount per well [pmol]: black, 10; gray, 1; white, 0.1). (C) *Tlr23479*^{-/-} Sirp^{high} cDCs were transfected with 100 ng per well of the *S. aureus* RNA preparations indicated or an ORN covering the SalII core sequence (10 pmol per well), either in the absence of (none) or upon preincubation for 20 min with 100 pmol per well antisense RNA ORN (SalIIas, +antisense). (D to F) Undifferentiated bone marrow cells were challenged with ORNs at the doses per well indicated in (D) and (E) or 100 pmol per well (F). (A to F) Cells were transfected [(A) Dotap, (B to F) Lyovec] for 16 hours with the indicated RNAs and ORNs. In each experiment, supernatants were assayed for nitrite content by Griess assay (A) or proinflammatory cytokine contents by bead assay (B and C) or ELISA (D to F). Each panel illustrates a representative result of three independent experiments and depicts means $\pm$ SD (error bars) of duplicate samples (A to E) or the mean $\pm$ SD of at least three independent experiments (F).

Fig. 4. TLR13 recognizes heat-inactivated *S. aureus* and ORNs mirroring bacterial 23S rRNA segments covering A2085/2058. (A) 5×10^5 *Tlr23479*^{-/-} macrophages were transfected with 50 pmol mRNA-specific siRNAs or scrambled control siRNA (scram.). After 48 hours, cells were challenged for 16 hours with 100 pmol per well ORN SaIII (black columns, bottom) or left untreated (white columns, bottom), and supernatants were analyzed by ELISA (bottom). Untreated cells were lysed to isolate mRNA, and levels of corresponding mRNAs were determined by reverse transcription polymerase chain reaction (top and middle). (B to E) HEK293 line cells were transfected with control, TLR2, or TLR13 expression and luciferase reporter plasmids. In general, cells were transfected with 15 ng empty vector (empty v.), 2 ng TLR2 (C), 15 ng TLR13 (D and E), or the amounts of TLR13 expression plasmid indicated in (B) and (C). At 24 hours posttransfection, cells were challenged with 10^9 , 10^8 , and 10^7 CFU/ml of hiSa (B); 10^9 CFU/ml hiSa (C); 100, 10, and 1 pmol per well ORN (C and D); 100 pmol per well ORN only (D and E); or 100 and 10 pmol per well ORN (E). Either 10 μ M of oligodeoxynucleotides (ODN, 1668 and 2006) only or 10 and 1 μ M of ODN was applied. ORN RNA40 was transfected with the reagent Dotap (E). After incubation for 16 hours, NF- κ B-driven relative



luciferase activity (rel. lucif. activ.) was analyzed. n.p., not performed; -, no challenge. (A to E) Each panel illustrates a representative result of three experiments and depicts means $\pm$ SD (error bars) of triplicate samples. (F) WT mice were challenged by i.v. injection of 10 nmol of ORN or ODN ($n = 9$ mice per group) in 200 μ l PBS or PBS alone (-). Serum was drawn 6 hours later and analyzed for IL-12p70 content by cytometric bead arrays (IL-12). Combined data of three experiments in which three mice per group were applied are shown as the mean of individual results.

(*S. aureus* 23S rRNA A2085G, mimicked by ORN Sa12s6G or Sa12s6U) failed to stimulate bone marrow cells (Fig. 3F and table S1) (18, 23). These findings suggest that molecular mechanisms rendering bacteria resistant to naturally occurring antibiotics also impede MyD88-dependent host recognition by an ill-defined endosomal TLR. To characterize the responsible TLR, we focused on TLR13, because analysis of *Tlr8*^{-/-} macrophages ruled out the involvement of TLR8. Specifically, WT and *Tlr8*^{-/-} macrophages exhibited comparable response to hiSa upon blockade of TLR7, TLR9, and TLR2. Moreover, responsiveness to 23S rRNA-derived SalII was similar (fig. S4A). Notably, small interfering RNA (siRNA)-driven suppression of TLR13 mRNA accumulation impaired the recognition of stimulatory ORNs such as SalII by *Tlr23479*^{-/-} macrophages (Fig. 4A). Although recognition of low doses of hiSa by *Tlr23479*^{-/-} macrophages treated with siRNA for TLR13 was strongly impaired, high-dose hiSa challenge activated not only control but also TLR13 siRNA-treated cells, presumably via unsuppressed TLR13 molecules (fig. S4B). In addition, knockdown of MAPK1 mRNA indicated involvement of MAPK1 in TLR13-driven signal transduction (Fig. 4A and fig. S4B). Furthermore, ectopic expression of TLR13 but not of CD14, TLR3, -7, -8, -9, or -12 conferred responsiveness of human embryonic kidney (HEK) 293 cells toward hiSa or the ORNs SalII, Sa23, Sa17, or Sa12 (Fig. 4, B to D, and fig. S4, C and D). Other ORNs such as RNA40 (TLR7 ligand) or CpG-containing oligodeoxynucleotides (ODNs) (TLR9 ligands) were inactive (Fig. 4E). Having identified the conserved 23S rRNA sequence “CGGAAAGACC” as a ligand for TLR13,

we set out to evaluate its importance in vivo. Therefore, we compared the cytokine storm induced by systemic application of TLR13-activating ORNs with that of TLR9-activating CpG-ODNs. Application of a nuclease-resistant phosphorothioate Sa19 variant (Sa19 PSO) in vivo triggered systemic proinflammatory cytokine release similar to that elicited by the PSO-CpG oligonucleotide 1668 (Fig. 4F and fig. S4, E and F). Consequently, systemic application of Sa19PSO to mice along with interferon- γ (IFN- γ) and D-galactosamine sensitization induced a fatal, septic shock-like syndrome in mice with functional TLR13 (WT and *Tlr23479*^{+/+}), whereas the *3D/Tlr24*^{-/-} mice that lack responsiveness to TLR13 were resistant (fig. S4G), concordant with the genotype-selective fatal pathology elicited by systemic challenge with hiSa (Fig. 1F). In contrast to the ORN Sa19, an ODN version of Sa19 (Sa19DNA, containing two CpG motifs) lacked TLR13 stimulatory activity but activated TLR9 (fig. S4F). Together, these data indicate that TLR13 functions as an important bacteria sensor by recognizing an ssRNA segment within the peptidyl transferase loop of bacterial 23S rRNA that binds antibiotics of the MLS group.

Our data unravel an unanticipated link between antibiotic resistance and evasion from TLR13 recognition, because 23S rRNA modifications generating resistance toward MLS antibiotics also camouflaged bacteria from TLR13 recognition. MLS antibiotic-producing bacteria such as *Saccharopolyspora erythraea* were possibly first to express erms (to resist their own antibiotics) (17). Erm expression plasmids might have been acquired from *S. erythraea* by staphylococci,

pneumococci, and mycobacteria (which seem to accompany or even correlate with the tuberculous property of the latter) (17, 24). Though macrolide resistance appears to be associated with fitness costs (21), the pathogenic recipients did gain invisibility to TLR13. We therefore speculate that widespread ancient antibiotic resistance (25) has subverted TLR13-driven antibacterial immune resistance, which may explain why TLR13 expression has been abandoned in certain mammalian species, including humans. If so, we anticipate that, in humans, the function of TLR13 has been replaced by an RNA-sensing PRR that is able to still recognize erythromycin resistance-forming RNA modifications.

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Acknowledgments: We thank S. Schimanski, J. Pätzold, R. Kassub, T. Scholtysik, and P. Juszczak, as well as C. Chebrolu for technical work; M. Freudenberg, J. Steinmann, and J. Kehrman for provision of mice and bacteria; R. Lang for advice; the German research foundation (Deutsche Forschungsgemeinschaft) for funding of KI 591/4-1 and GK1045 (to C.J.K.) and BA 1618/5-1 (to S.B.), as well as to U.K.; and the Japanese Government for funding to S.A. and T.K. The Behring Röntgen Stiftung funded grant 56-0034 (to S.B.). The Univ. of Duisburg-Essen (C.J.K.), Bavarian Nordic GmbH (H.H.), and the Philipps Univ. of Marburg (S.B.) filed a patent on TLR13 activators (U.S. Patent 61/597,063; agonists and antagonists of TLR13). H.W. and S.B. had an advisory contract with Coley Pharmaceuticals/Pfizer that ended in 2010. The data presented in this paper are tabulated in the main paper and in the supplementary materials.

Supplementary Materials

www.sciencemag.org/cgi/content/full/science.1220363/DC1
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10 February 2012; accepted 3 July 2012
Published online 19 July 2012;
10.1126/science.1220363

Compartmentalized Control of Skin Immunity by Resident Commensals

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Intestinal commensal bacteria induce protective and regulatory responses that maintain host-microbial mutualism. However, the contribution of tissue-resident commensals to immunity and inflammation at other barrier sites has not been addressed. We found that in mice, the skin microbiota have an autonomous role in controlling the local inflammatory milieu and tuning resident T lymphocyte function. Protective immunity to a cutaneous pathogen was found to be critically dependent on the skin microbiota but not the gut microbiota. Furthermore, skin commensals tuned the function of local T cells in a manner dependent on signaling downstream of the interleukin-1 receptor. These findings underscore the importance of the microbiota as a distinctive feature of tissue compartmentalization, and provide insight into mechanisms of immune system regulation by resident commensal niches in health and disease.

Mammals and their microbiota have formed an evolutionary partnership that is critical for metabolism, tissue development, and host defense (1–3). In particular, the gut flora has been implicated in intestinal

immune tissue development and function, as well as in promoting systemic inflammation in the context of autoimmunity and infection (1, 4–8). Despite our growing understanding of the consequences of this host-microbe alliance for intes-

tinal immune function, the degree to which the gut flora contributes to immunity at distal sites remains unclear.

The skin represents the primary interface between the host and the environment. Microbial

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profiling has revealed the presence of highly diverse commensal communities along distinct topographical skin sites (9, 10). Moreover, cutaneous inflammatory disorders such as psoriasis, atopic dermatitis, and rosacea have been associated with dysbiosis in the cutaneous microbiota (11, 12). Indeed, microbial products from skin commensals are known to exert immunoregulatory effects (13).

In humans, commensals reside in defined niches such as hair follicles and sebaceous glands (14). Microbes or microbial products densely coat the outer skin layer, hair follicles, and sebaceous glands in mice raised under specific pathogen-free (SPF) conditions (Fig. 1A). As previously described (15), the skin tissue of SPF mice contains a high frequency of Foxp3⁺ regulatory T cells (T_{regs}) (Fig. 1B and fig. S1, A and B). This compartment is also home to T cell receptor (TCR) αβ⁺ T cells with the potential to produce substantial amounts of the cytokines interferon-γ (IFN-γ) or interleukin-17A (IL-17A), the latter produced by CD4⁺ and CD4⁺ CD8⁺ T cells (Fig. 1, B and C, and fig. S1, A to C), as well as TCRγδ^{low} dermal T cells (Fig. 1C and fig. S1, A and B) (16). Microbe-derived products were undetectable in the skin of germ-free (GF) mice born and raised in aseptic conditions (Fig. 1A). In the intestine, the balance between effector and regulatory T lymphocytes is tightly controlled by commensal signals (17–19). Similarly, we observed a significant reduction in IFN-γ and IL-17A production by αβ T cells, and IL-17A by γδ^{low} T cells, in skin tissue of GF mice relative to SPF mice (Fig. 1, B and C, and fig. S1D). Concomitantly, the frequency and absolute numbers of cutaneous Foxp3⁺ T_{regs} were increased in the absence of commensals (Fig. 1B and fig. S1D). The influence of commensals on T cell subset frequencies did not extend to draining lymph nodes (fig. S2A).

Defects in effector responses, previously observed in the gut of GF mice, have in part been attributed to impaired development of tissue and associated lymphoid structures (5). Surveys of skin homing receptors on peripheral T cells and frequencies of cutaneous γδ or αβ T cells, eosinophils, mast cells, and dendritic cell subsets revealed no differences between GF and SPF mice (fig. S2, B to F). In line with previous analyses (20, 21), the cellularity and architecture of skin-draining lymph nodes were comparable between SPF and GF mice (fig. S2, C and G). Thus, effector T cell functional potential in the skin is critically dependent on signals from the commensal microbiota and independent of developmental defects.

Differences in dermal T cell effector profiles in GF mice may result from a lack of signals from the gut flora, which have been implicated in the control of both local and systemic immune responses (4, 7, 8, 22). In particular, mice harboring segmented filamentous bacteria (SFB) in their gastrointestinal (GI) tract display increased intestinal levels of CD4⁺ T cell-derived IL-17A and

IFN-γ relative to mice devoid of such bacteria (23, 24). Consistent with previous reports, monoassociation of GF mice with SFB reconstituted IL-17A and IFN-γ to levels observed in the GI tract of SPF mice (fig. S3, A and B). In contrast, the presence of SFB did not restore effector cytokine production or alter T_{reg} frequencies in the skin (fig. S3, A to C). To further address this point, we used a well-established oral antibiotic regimen known to decrease the density of intestinal flora (17, 19, 25). After oral antibiotic treatment, the overall density and composition of gut microbiota, but not skin microbiota, were profoundly altered (17) (Fig. 2A and fig. S3, D and E). Alterations of gut flora had no effect on the capacity of T cells to produce inflammatory cytokines (Fig. 2B).

We next monoassociated GF mice with the skin commensal *Staphylococcus epidermidis* (9). Colonization with this single commensal organism was sufficient to rescue IL-17A production in the skin but not the gut (Fig. 2, C and D). Furthermore, treatment with oral vancomycin to prevent *S. epidermidis* from colonizing the gut had no effect on this bacterium's ability to rescue cutaneous IL-17A levels (fig. S3F). Together, our results show that resident bacteria are necessary

to drive effector T cell function in the skin, and that fluctuations in the gut microbiota have no direct effect on cutaneous immune homeostasis.

To evaluate the functional consequences of skin commensals on local immunity, we used a model of dermal infection induced by the protozoan parasite *Leishmania major* (26). Protective immunity to *L. major* is dependent on T cell-derived IFN-γ (26). Previous work demonstrated impaired parasite control in the absence of commensals upon intramuscular parasite delivery (27). Relative to SPF mice, GF mice infected intradermally with *L. major* manifested smaller lesions with reduced edema and necrosis (Fig. 3, A and B). Effector responses were severely impaired in GF mice, as evidenced by a reduction in *Leishmania*-specific IFN-γ and production of the pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) by cutaneous T cells (Fig. 3, C to E, and fig. S4A). Altered immunity and parasite control were not associated with increased T_{reg} frequencies or IL-10 responses (fig. S4, B and C).

To corroborate a role for skin commensals in promoting immunity to *L. major*, we monoassociated GF mice with *S. epidermidis* at the time of infection. Colonization of GF skin with

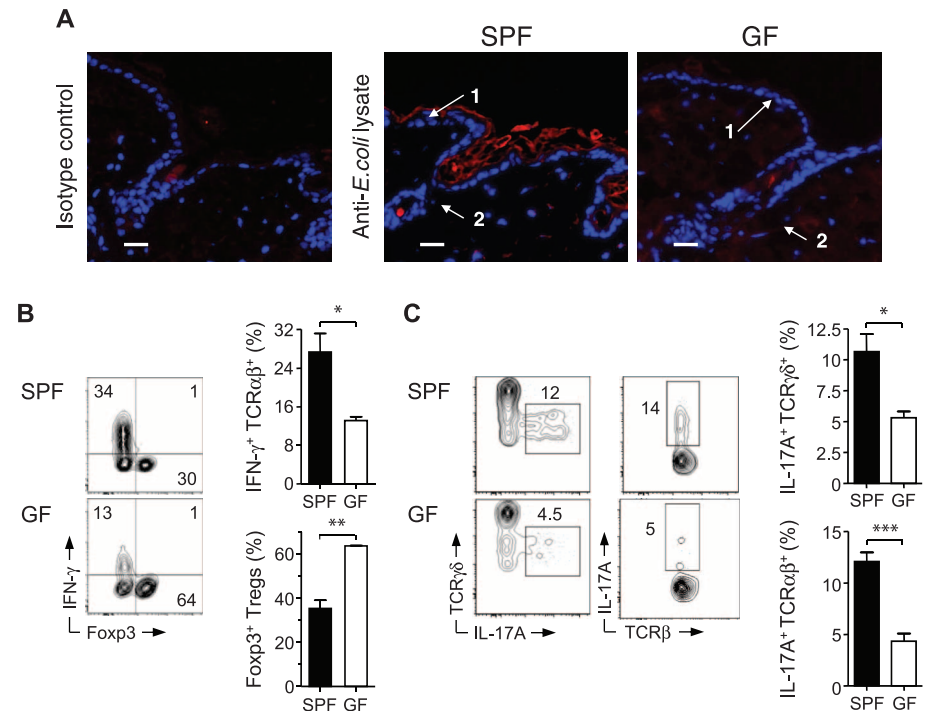


Fig. 1. Commensal microbiota control the balance of effector and regulatory T cells in the skin tissue. **(A)** Immunofluorescence labeling of bacterial products in interfollicular keratinocytes (1) and hair follicles (2) from skin tissue of SPF and GF mice. Representative images show naïve skin stained with anti-*E. coli* lysate antibody (red) or isotype control and Hoechst (blue); scale bars, 25 μm. **(B)** Representative flow cytometric plots and summarized bar graphs of IFN-γ and Foxp3 expression by live CD45⁺ TCRβ⁺ cells extracted from skin tissue of SPF and GF mice after stimulation with phorbol myristate acetate (PMA) and ionomycin. Graphs show means ± SEM of three or four mice (**P* < 0.05, ***P* < 0.005). Results are representative of three experiments. **(C)** Representative flow cytometric plots and summarized bar graphs of IL-17A expression in live CD45⁺ TCRγδ⁺ or CD45⁺ TCRαβ⁺ cells from skin tissue of SPF and GF mice after stimulation with PMA and ionomycin. Graphs show means ± SEM of three or four mice (**P* < 0.05, ****P* < 0.0005). Results are representative of three experiments.

Fig. 2. Distinct commensal niches control T cell cytokine production in the gut and skin. **(A)** Taxonomic classifications at the phylum level for 16S ribosomal RNA gene sequence data clustered at 97% identity from skin tissue and fecal pellet of control mice and mice treated with oral antibiotic cocktail (ATB) for 4 weeks. Each column represents an individual mouse. **(B)** Assessment of IFN- γ production in live CD45 $^{+}$ TCR β^{+} cells and IL-17A production in live CD45 $^{+}$ cells from skin and intestine of mice treated with oral antibiotic cocktail or water (Ctrl) for 4 weeks. Graphs show means $\pm$ SEM of four mice (** P < 0.005, *** P < 0.0005; ns, not significant). Results are representative of two or three experiments. **(C and D)** Flow cytometric analysis of IL-17A production in live CD45 $^{+}$ TCR β^{+} cells from the gut and skin of SPF mice, GF mice, and GF mice monoassociated with *S. epidermidis* (GF + *S.epi*) for 2 to 3 weeks. Graphs show means $\pm$ SEM of three to five mice (** P < 0.005). Results are representative of two experiments.

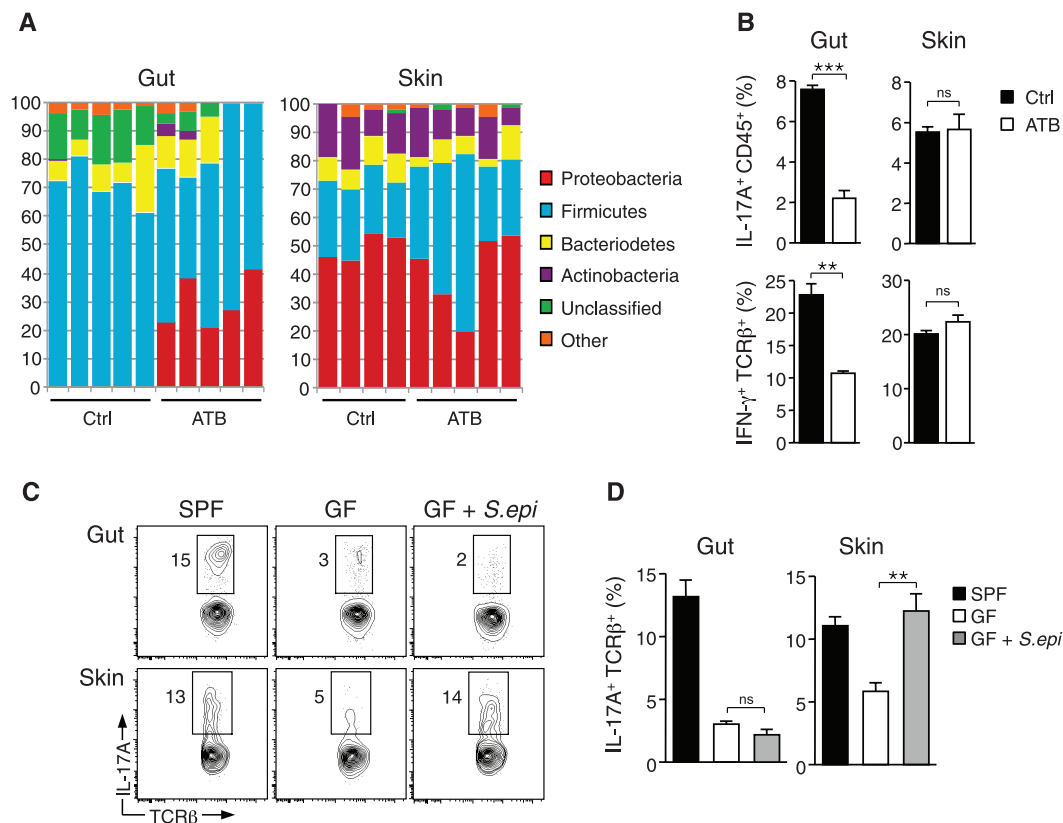
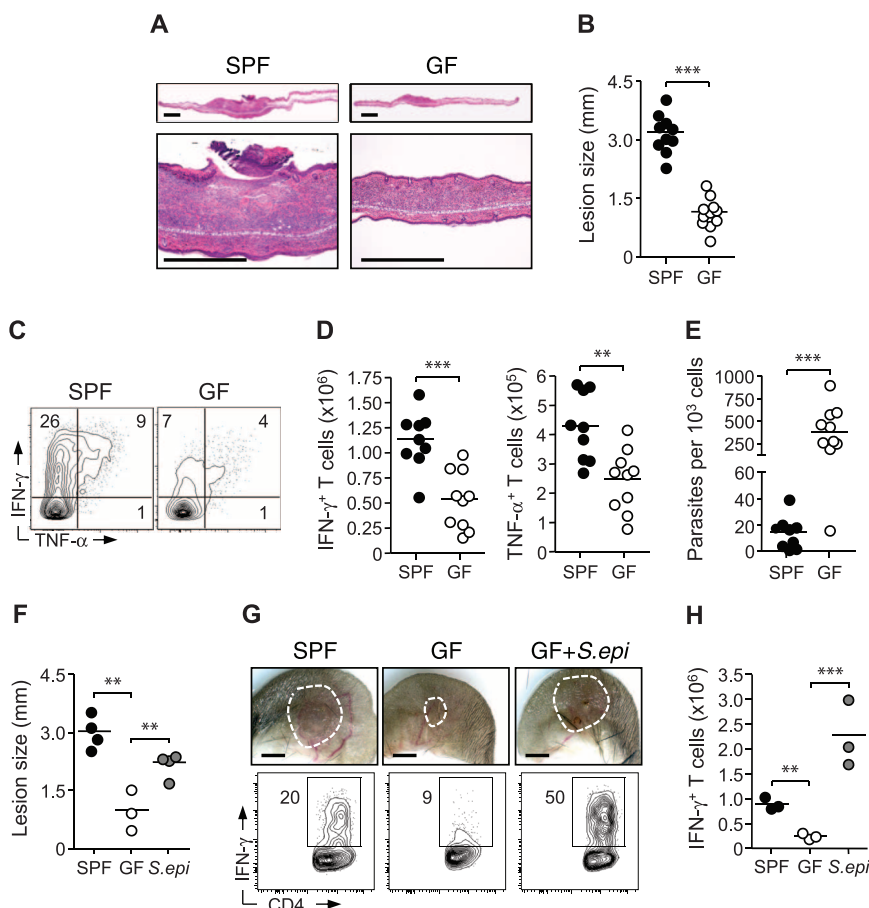


Fig. 3. Cutaneous commensals drive immunity and promote pathology in *L. major* infection. **(A)** Histopathological comparison of ear pinnae skin lesions from *L. major*-infected SPF and GF mice. Scale bars, 500 μ m. **(B)** Assessment of lesion size in SPF and GF mice. Each data point represents an individual mouse (*** P < 0.0005). **(C and D)** Flow cytometric analysis of *Leishmania* antigen-specific IFN- γ and TNF- α production by TCR β^{+} CD4 $^{+}$ dermal cells from *L. major*-infected SPF and GF mice. Each data point represents an individual mouse (** P < 0.005, *** P < 0.0005). Results are representative of three experiments. **(E)** Number of *L. major* parasites per 1000 nucleated cells from dermal lesions of infected SPF and GF mice. Each data point represents an individual mouse (*** P < 0.0005). **(F)** Assessment of lesion size in SPF mice, GF mice, and GF mice monoassociated with *S. epidermidis* (*S.epi*). Each data point represents an individual mouse (** P < 0.005). Results are representative of two experiments. **(G and H)** Representative images of *L. major* skin lesions and analysis of IFN- γ production by live TCR β^{+} CD4 $^{+}$ cells from SPF mice, GF mice, and GF mice monoassociated with *S. epidermidis*. Each data point represents an individual mouse (*** P < 0.0005). Results are representative of two experiments.



this commensal was sufficient to rescue protective immunity in these animals (Fig. 3, F to H, and fig. S4D). Monoassociation of GF mice with *S. epidermidis* also restored pathology with increased necrosis; this finding supports the idea that pathological consequences of this skin infection depend on the presence of commensals rather than the parasite (Fig. 3, F and G). Further implicating cutaneous commensals in the direct control of skin immunity and inflammation, oral vancomycin treatment of GF animals associated with *S. epidermidis* had no influence on immunity and pathology during *L. major* infection (fig. S4, E and F).

The dominant cytokine signals that drive effector cytokine production by T cells in the skin have not yet been elucidated. By screening mice that were deficient in factors known to drive IL-17A production, we determined that IL-1R1 and its downstream signaling complex MyD88, but not IL-23R or IL-6, played a dominant role in controlling the production of IL-17A, but not IFN- γ , by cutaneous T cells (Fig. 4A and fig. S5, A to C). Toll-like receptor 2 (TLR2), which also relies on MyD88-dependent signaling, is required to sense by-products from *S. epidermidis* during skin inflammation (13). However, mice deficient in TLR2 as well as TLR3, 5, and 9 did not display a reduction in T cells or cytokine production relative to control mice under steady-state conditions (fig. S5D). Hence, our data support the idea that the defect in IL-17A observed in MyD88-deficient mice is primarily a consequence of altered IL-1R1-mediated signaling. Consistent with a partitioning of the dominant signals controlling the skin and gut environment, MyD88 or IL-1R1 deficiency had no impact on T cell potential to produce IL-17A in the intestine (Fig. 4A).

Both $\alpha\beta$ and $\gamma\delta$ effector T cells are known to express IL-1R1 and to respond directly to IL-1 (28–31). We purified skin lymphocytes and stimulated them via their TCR in the presence of IL-1 α , IL-1 β , or IL-6. Under these conditions, IL-1 α and IL-1 β , but not IL-6, potently increased the capacity of T cells to release IL-17A (Fig. 4B and fig. S5E). Thus, T cells that reside at dermal sites can be functionally tuned by the local cytokine milieu, and in particular by IL-1.

We next explored the possibility that IL-1 signaling may be diminished in the absence of commensals. Indeed, IL-1 α production by cutaneous cells was significantly reduced in GF mice relative to SPF mice, and monoassociation of GF mice with *S. epidermidis* restored the production of this cytokine (Fig. 4C). Additionally, keratinocytes from GF mice displayed increased levels of the IL-1 receptor antagonist (IL-1ra) mRNA relative to SPF mice, indicating that commensals control various aspects of functional IL-1 signaling (fig. S5F). Complementing these observations, the addition of *S. epidermidis* to GF mice significantly reduced IL-1ra from cutaneous cells (fig. S5G). Therefore, resident commensals

are required for optimal IL-1 signaling in the skin, which in turn promotes local effector responses.

To ascertain the importance of the MyD88 pathway in hematopoietic cells, we generated mixed bone marrow chimeras using MyD88/TRIF knockout

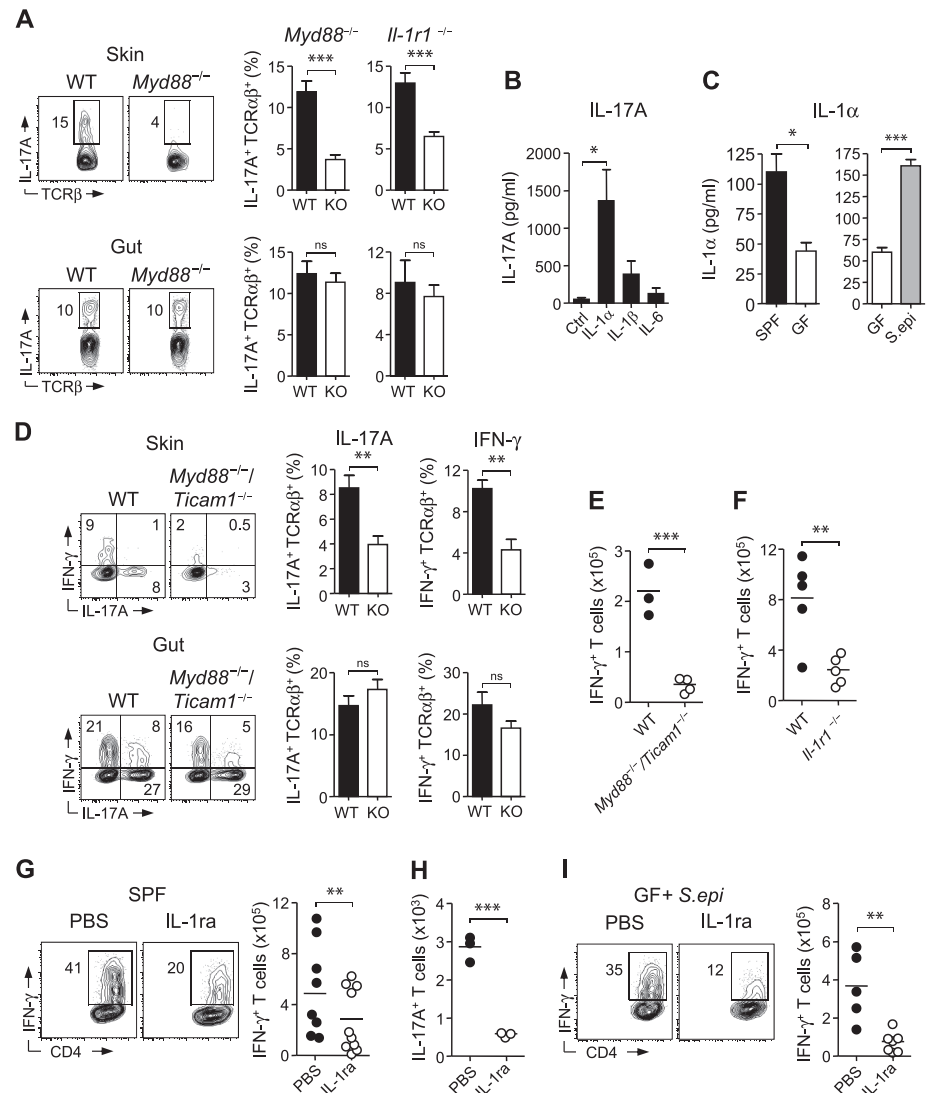


Fig. 4. Skin-resident commensals modulate dermal T cells in a manner dependent on IL-1 and MyD88. (A) Flow cytometric analysis of IL-17A production by live CD4⁺ TCR β ⁺ cells in skin and intestine of age-matched *Myd88*^{-/-} and *Il1r1*^{-/-} mice. WT, wild type; graphs show means $\pm$ SEM of three or four mice ($***P < 0.0005$). Results are representative of two or three experiments. (B) IL-17A production from purified skin CD4⁺ TCR β ⁺ T cells cultured in vitro in the presence of anti-CD3 and either IL-1 α , IL-1 β , or IL-6. Graphs represent the mean of three experimental groups $\pm$ SEM ($*P < 0.05$). Results are representative of three experiments. (C) Spontaneous release ($\pm$ SEM) of IL-1 α from skin-derived cells of SPF mice, GF mice, and GF mice monoassociated with *S. epidermidis* (*S.epi*) as measured by enzyme-linked immunosorbent assay ($*P < 0.05$, $***P < 0.0005$). (D) Comparative assessment of IFN- γ and IL-17A production from WT and *Myd88*^{-/-}/*Ticam1*^{-/-} TCR β ⁺ cells from mixed bone marrow chimeric mice. Bar graphs show frequency ($\pm$ SEM) of cytokine production by WT and knockout TCR β ⁺ cells. Results are representative of two experiments in the skin and one experiment in the gut ($**P < 0.005$). (E and F) Analysis of *L. major*-specific IFN- γ production by TCR β ⁺ CD4⁺ T cells from WT and *Myd88*^{-/-}/*Ticam1*^{-/-} mice. Results are representative of two experiments. (G) Flow cytometric assessment of *L. major*-specific IFN- γ production from TCR β ⁺ CD4⁺ T cells from the skin of SPF animals treated with either IL-1ra or phosphate-buffered saline (PBS). Results are a compilation of two experiments. (H) Number of CD4⁺ TCR β ⁺ IL-17A⁺ T cells from the skin of GF mice monoassociated with *S. epidermidis* treated with either IL-1ra or PBS. Results are representative of one experiment. (I) *L. major*-specific IFN- γ produced by TCR β ⁺ CD4⁺ T cells from the skin of GF mice monoassociated with *S. epidermidis* and treated with either IL-1ra or PBS. Results are representative of two experiments. For (E) to (I), each data point represents an individual mouse ($**P < 0.005$, $***P < 0.0005$).

mice that phenocopied MyD88 knockout animals in their cutaneous IL-17A deficiency. (fig. S5, A, H, and I). Irradiation required for generating chimeras induces inflammation and homeostatic proliferation of T cells. Under these inflammatory conditions, MyD88 signaling in hematopoietic cells was required for the production of both IFN- γ and IL-17A in the skin, but not in the gut (Fig. 4D). Furthermore, mice deficient in MyD88 or IL1R1 displayed impaired effector responses and parasite control during *L. major* infection, as did mice treated with IL1ra (Fig. 4, E to G, and fig. S5J).

These results support the idea that defects in T cell function at steady state or during inflammation result from an impaired dialogue with skin commensals. To functionally link commensally driven IL-1 signaling to T cell immunity in the skin, we treated GF mice monoassociated with *S. epidermidis* with IL-1ra at steady state and during *L. major* infection. Neutralizing IL-1 activity in these animals hindered this bacterium's ability to rescue IL-17A at steady state and to promote *L. major*-specific IFN- γ during infection (Fig. 4, H and I). Thus, via their capacity to control IL-1 signaling, skin commensals promote effector T cell responses according to local inflammatory cues. On the basis of the pleiotropic role of IL-1, this effect may result from direct IL-1 signaling in T cells and/or modulation of various innate inflammatory cells such as neutrophils (32).

Our results indicate that resident commensals are necessary for optimal skin immune fitness. Specifically, we find that cutaneous commensals exert their effect by augmenting IL-1 signaling and amplifying responses in accordance with the local inflammatory milieu. The IL-1 pathway is an evolutionarily conserved arm of the innate

immune system that may have arisen as an early mediator of host skin-commensal cross talk. This pathway is also linked to a multitude of chronic inflammatory disorders such as arthritis and asthma. Moreover, IL-1 has been implicated in the etiology and pathology of psoriasis and other cutaneous disorders (33). Thus, via their capacity to promote IL-1 signaling and consequently effector T cell function, skin commensals are likely important drivers and amplifiers of skin pathologies. Understanding the role of the skin microbiota in maintaining tissue function is not only of primary importance for human health, but will also lead to the development of more rational tissue-specific adjuvants and vaccine approaches.

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Acknowledgments: Supported by the Division of Intramural Research of the National Institute of Allergy and Infectious Diseases (NIAID) and by the National Human Genome Research Institute, the National Cancer Institute, NIH grant F30 DK094708 (S.S.), and the Human Frontier Science Program (C.W.). We thank the NIAID gnotobiotic facility staff, in particular C. Acevedo and D. Trageser-Cesler; K. Holmes and the NIAID sorting facility; K. Becht, B. Kelsall, T. Tamachi, C. Brown, J. Benchley, A. MacDonald, L. Mijares, K. Shenderov, and A. Sher; and Yakult Central Institute for Microbiological Research for sharing SFB via a material transfer agreement. DNA sequence data are available via GenBank (accession no. SRS345653). The data reported in this paper are tabulated in the main paper and in the supplementary materials.

Supplementary Materials

www.sciencemag.org/cgi/content/full/science.1225152/DC1
Materials and Methods
Figs. S1 to S5
References (34–38)

24 May 2012; accepted 28 June 2012
Published online 26 July 2012;
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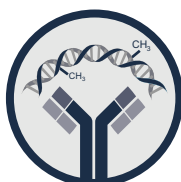
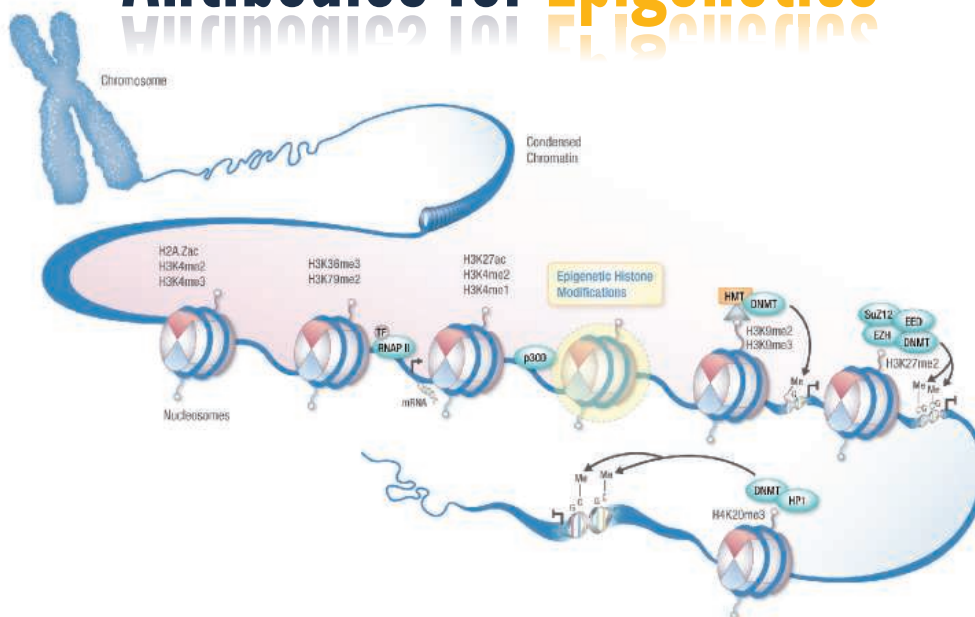
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LIST OF PROTEINS

4-1BBL	Caspase-3	sFlt-1 (D3)	IL-2	MEC	sRANK
4-1BB Receptor	Caspase-6	sFlt-1 (D4)	IL-3	Mek-1	sRANKL
6 Ckine	CD4	sFlt-1 (D5)	IL-4	MIA	RANTES
ACAD8	CD14	sFlt-1 (D7)	sIL-4 Receptor	Midkine	RELM- α
ACAT2	CD22	Flt3-Ligand	IL-5	MIG / CXCL9	RELM- β
gAcrp30/Adipolean	CD40 Ligand / TRAP	sFlt-4	IL-6	MIP-1 α / CCL3	Resistin
Activin A	CD95 / sFas Ligand	sFlt-4/ Fc Chimera	sIL-6 Receptor	MIP-1 β / CCL4	RPTP β
ACY1	CD105 / Endoglin	Follistatin	IL-7	MIP-3 / CCL23	RPTP γ
ADAT1	CHIPS	FSH	IL-8 (72 a.a.)	MIP-3 α / CCL20	RPTP μ
Adiponectin	CNTF	Fractalkine/ CX3C	IL-8 (77 a.a.)	MIP-3 β / CCL19	SCF
ADRP	Collagen	G-CSF	IL-9	MIP-4 (PARC) / CCL18	SCGF- α
AITRL	CREB	α -Galactosidase A	IL-10	MIP-5 / CCL15	SCGF- β
Akt1	CTACK/CCL27	Galectin-1	IL-11	MMP-3	SDF-1 α
Alpha-Feto Protein (AFP)	CTGF	Galectin-3	IL-12	MMP-7	SDF-1 β
Alpha-Galactosidase A	CTGFL/WISP-2	Gastrointestinal CA	IL-13	MMP-13	Secretin
Angiopoietin-1 (Ang-1)	CTLA-4/Fc	GCP-2	IL-13 analog	Myostatin	SF20
Angiopoietin-2 (Ang-2)	CXCL16	GDF-3	IL-15	Nanog	SHP-2
Angiostatin K1-3	Cytokeratin 8	GDF-9	IL-16 (121 a.a.)	NAP-2	STAT1
Annexin-V	DEP-1	GDF-11	IL-16 (130 a.a.)	Neurturin	c-Src
apo-SAA	Desmopressin	GDNF	IL-17	NFAT-1	TACI
Apolipoprotein A-1	Disulfide Oxidoreductase	GLP-1	IL-17B	beta-NGF	TARC
Apolipoprotein E2	E-selectin	Glucagon	IL-17D	NOGGIN	TC-PTP
Apolipoprotein E3	ECGF	Goserelin	IL-17E	NOV	TECK
Apolipoprotein E4	EGF	GM-CSF	IL-17F	NP-1	TFF2
APRIL	Elafin/SKALP	GPBB	IL-19	NT-1/BCSF-3	TGF- α
Artemin	EMAP-II	GRO α	IL-20	NT-3	TGF- β 1
ATF2	ENA-78	GRO β	IL-22	NT-4	TGF- β 2
Aurora A	Endostatin	GRO γ	IL-31	Ocreotide	TGF- β 3
Aurora B	Enteropeptidase	GRO/MGSA	Insulin	Oncostatin M	Thymosin α 1
BAFF	Eotaxin	Growth Hormone	IP-10	Osteoprotegerin (OPG)	sTIE-1/Fc Chimera
BAFF Receptor	Eotaxin-2	Growth Hormone BP	JE	OTOR	sTIE-2/Fc Chimera
BCA-1 / BLC / CXCL13	Eotaxin-3 (TSC)	GST-p21/WAF-1	JNK2a1	Oxytocin	TL-1A
BCMA	EPHB2	HB-EGF	JNK2a2	p38- α	TNF- α
BD-1	EPHB4	HCC-1	KC / CXCL1	Parathyroid Hormone	TNF- β
BD-2	Eptifibatide	HGF	KGF	PDGF-AA	sTNFR1
BD-3	Erk-2	Histidyl-tRNA synthetase	L-asparaginase	PDGF-AB	sTNFR2
BDNF	Erythropoietin (EPO)	Histrelin	LAG-1	PDGF-BB	TPO
Bivalirudin	Exodus-2	HRG1- β 1	LALF Peptide	Persephin	TRAIL/Apo2L
BMP-2	Fas Ligand	I-309	LAR-PTP	PF-4	sTRAIL R-1 (DR4)
BMP-4	Fas Receptor	I-TAC	LC-1	PIGF-1	sTRAIL R-2 (DR5)
BMP-7	FGF-1 (acidic)	IFN- α	LBP	PIGF-2	TSH
BMP-13	FGF-2 (basic)	IFN- α A	LD-78 β	PKA α -subunit	TSLP
sBMPR-1A	FGF-4	IFN- α 2a	LDH	PKC- α	TWEAK
Brain Natriuretic Protein	FGF-5	IFN- α 2b	LEC/NCC-4	PKC- γ	TWEAK Receptor
BRAK	FGF-6	IFN- β	Leptin	Pleiotrophin	Urokinase
Breast Tumor Antigen	FGF-7/ KGF	IFN- γ	LIGHT	PLGF-1	VEGF121
C5a	FGF-8	IFN-Omega	LIX	Polymyxin B (PMB)	VEGF145
C5L2 Peptide	FGF-9	IGF-I	LKM	PRAS40	VEGF165
C-10	FGF-10	IGF-II	LL-37	PRL-1	VEGF-C
C-Reactive Protein	FGF-16	proIGF-II	Lymphotactin	PRL-2	VEGF-C I525
C-Src	FGF-17	IGFBP-1	sLYVE-1	PRL-3	EG-VEGF
Calbindin D-9K	FGF-18	IGFBP-2	M-CSF	Prokineticin-2	VEGF-E
Calbindin D-28K	FGF-19	IGFBP-3	MCP-1 (MCAF)	Prolactin	HB-VEGF-E
Calbindin D-29K	FGF-20	IGFBP-4	MCP-2	Protirelin	sVEGFR-1
Calmodulin	sFGFR-1 (IIIc) / Fc Chimera	IGFBP-4	MCP-3	PTHrP	sVEGFR-2
Calcitonin Acetate	sFGFR-2 (IIIc) / Fc Chimera	IGFBP-5	MCP-4	PTP1B	sVEGFR-3
Carbonic Anhydrase III	sFGFR-3 / Fc Chimera	IGFBP-6	MCP-5	PTP-IA2	WISP-1
Carcino-embryonic Antigen	sFGFR-4 / Fc Chimera	IGFBP-7	MDC (67 a.a.)	PTP-MEG2	WISP-2
Cardiotrophin-1	sFlt-1 (native)	IL-1 α	MDC (69 a.a.)	PTP-PEST	WISP-3
		IL-1 β	MDH		WNT-1

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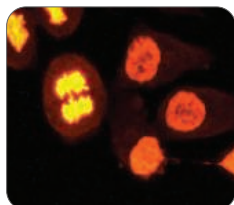


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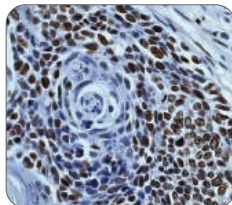
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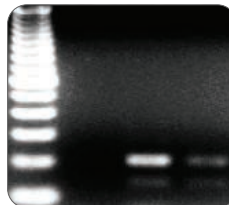
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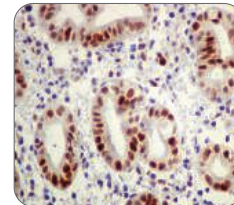
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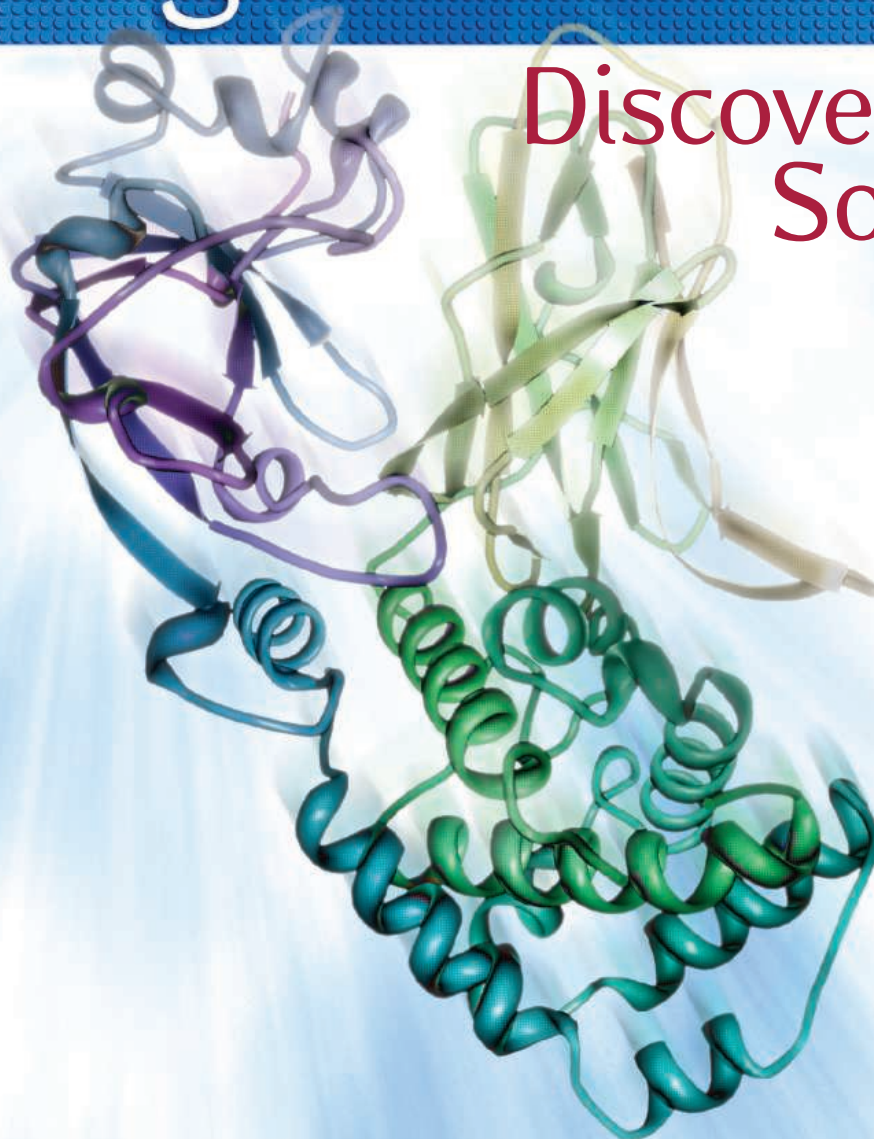
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LOCATION: Gyro King
ARTICLE: *Cavemen Craved Carbs, Too*
DATE: Sep 21, 1:13pm

LOCATION: Hemlock Bar
ARTICLE: *Quantum Simulation of Frustrated Classical Magnetism in Triangular Optical Lattices*
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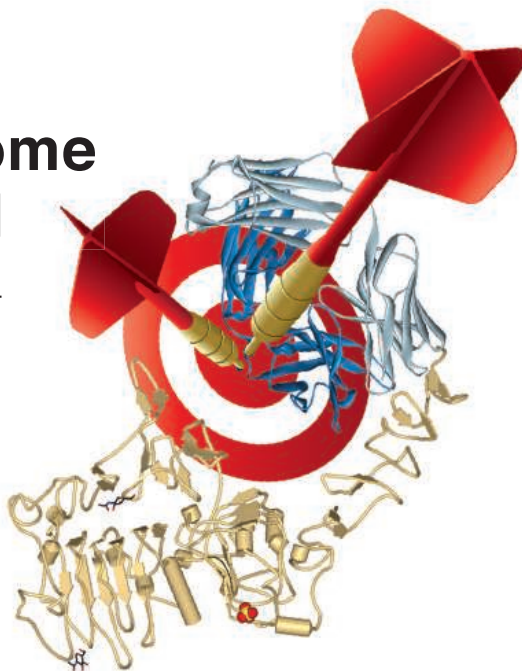
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Proteomics

Panning the Proteome for Biomarker Gold

Targeted proteomics is homing in on promising biomarkers to help screen for cancer and guide patient treatment, but much work still needs to be done to validate these biomarkers and develop technology capable of bringing them to the clinic. **By Anne Harding**



“Once we know what to look for, instead of trying to measure thousands of proteins, if we’ve got a specific target list we can develop assays that home in on these proteins.”

Testing breast tumors for certain cell surface receptors is now a routine part of cancer treatment. Patients with the HER2/neu receptor, for example, tend to have a more aggressive disease and will be given the targeted drug Herceptin. Tumors that carry estrogen receptors—about 70 percent of all breast cancers—will respond to tamoxifen and other selective estrogen receptor modulators.

But one day, if the promise of proteomics is realized, cancer treatments could be tailored to a patient by assessing the entire proteome of a tumor cell, rather than just one or two receptors. “We envision that a breast cancer patient could come to the doctor in the morning and get the proteomics analysis, and maybe get the results in the afternoon,” says Matthias Mann, an expert in proteomics and mass spectrometry and director at the **Max Planck Institute of Biochemistry** in Martinsried, Germany. “The question is very open of whether this can be done or not. Whether or not it would work, it would be so important that it is incumbent on us that we should try.”

To fulfill the promise of clinical proteomics, investigators must overcome multiple hurdles, including the difficulty of obtaining adequate samples, a dearth of antibodies—and funding—for validation studies, and limitations with the accuracy and throughput of available technologies, such as mass spectrometry. But bit by bit, some of these hurdles are being overcome as basic scientists develop innovative strategies for data analysis as ever more powerful and sensitive mass spectrometers hit the market.

SLOW PROGRESS

When investigators first began looking into clinical proteomics, technical limitations meant they could only measure a few hundred proteins at once, while flawed data analysis usually yielded “biomarkers” that didn’t turn out to reveal anything at all. Although there have been over 10,000 publications on biomarker discovery with proteomics, the U.S. Food and Drug Administration (FDA) has only approved a single proteomics-based diagnostic test. Vermillion’s OVA1, cleared by the FDA in 2009, measures

five different proteins in the blood, and is not used to screen for ovarian cancer, but to help evaluate whether an ovarian mass is benign or malignant prior to surgery.

“What you’ve seen in the industry is a lot of great technological developments, but in terms of locking down and then validating there are very few of these tests that have really made it through that process and ultimately been commercialized and validated in clinical practice,” says Paul Beresford, vice president of business development and strategic marketing at **Biodesix**, a molecular diagnostic company.

The classic approach to asking clinical questions with proteomics has been to look at tissue or body fluid samples from patients with a given disease, which is verified by pathology, and compare them to a control group. “One of the critical issues is, of course, that measuring only a couple of hundred samples, control and case, would need to be done at a high enough throughput, and these measurements must be reproducible,” says Ruedi Aebersold, a professor of systems biology at the **Swiss Federal Institute of Technology** in Zurich. “The conventional mass spectrometry technologies have great difficulty achieving these goals.”

And some say that testing hundreds of samples won’t be enough. “There’s a general conclusion coming out of the real diagnostics industry that if you can’t look at 1,500 to 2,000 samples there simply isn’t any way you could know whether a biomarker is relevant to clinical practice,” says Leigh Anderson, CEO of **SISCAPA Assay Technologies**, which develops assays for analyte enrichment at the peptide level for use with multiple reaction monitoring (MRM) mass spectrometry. The company

UPCOMING FEATURES

Genomics: Epigenetics/Epigenomics—October 26

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has 12 assays in production and 54 in development.

“Clinical proteomics has been done for 50 years; people have had their serum proteins measured to detect disease for decades,” says Stephen Kron, a professor of molecular genetics and cell biology at **The University of Chicago**. “But the question is: Can you do better than the proteomics we have now? Rather than just incrementally better, can you do better than ELISA? Can you make systems that are robust and highly multiplexed? The key thing is, there’s no one answer, and the current methods are inadequate despite all of us using them.”

NEEDLES IN HAYSTACKS

The vast dynamic range of the human proteome—especially the human plasma proteome—has been one of the biggest challenges in using global proteomics for biomarker discovery. The concentration of the lowest abundant proteins found in plasma, among which many investigators believe biomarker “gold” is most likely to be found, is 10 to 12 orders of magnitude lower than the concentration of the most abundant plasma protein, albumin. “That exceeds the dynamic range of pretty much any instrument we’re trying to use to measure proteins,” says Steven Skates, an associate professor of medicine at **Harvard Medical School and Massachusetts General Hospital (MGH)** who is studying early detection of ovarian cancer.

In collaboration with a team of investigators from MGH, the Broad Institute, and the Dana Farber Cancer Institute, Skates has been using several different strategies to expand the dynamic range of his experimental techniques. “One possibility is to use specimens that have a much more concentrated biomarker content,” he explains. “It might be tumor tissue itself or fluid that arises from the tumor, but isn’t diluted as it would be in the blood.” He is currently looking at fluid from ovarian cysts, which potentially contain cancer-related proteins at a thousand-fold higher concentration than plasma.

Many investigators, including Skates, use fractionation to look at less abundant proteins; yet another strategy is to deplete higher abundance proteins from a sample.

“With a combination of depleting abundant proteins, high fractionation, and starting with a more concentrated biomarker source than blood, you’ve got about seven orders of magnitude that you’re crossing, and then with three orders of magnitude from the instrument, that gives you 10 orders of magnitude,” Skates adds. “We’re just at the tip of where we think the biomarkers are.”

TARGETED PROTEOMICS

Leaders in proteomics now agree that the “shotgun” or “brute force” approach to searching for biomarkers is an incomplete paradigm that falls short of the clinical goal. “I’m personally frustrated that we have been attempting to play with the technology over the past 15 years to find a shortcut,” says Anderson. “But at the end of the day, you’re going to have to commit to the details of specific hypotheses and make some serious measurements of the few proteins that make a difference.”

That’s where targeted mass spectrometry comes in. “Once we know what to look for, instead of trying to measure thousands of proteins, if we’ve got a specific target list we can develop assays that home in on these proteins,” Skates says.



“You could think of taking a digital photo of a crowd at a football match, and then also having photos of individuals who have been previously identified.” You could then use the individual portraits to determine who was present in the group photo.

“One of the big rate limiting steps is going from those candidates we identify with global proteomics techniques to developing assays for accurate measurement of proteins in the blood,” he adds. “Developing assays is very difficult because the blood is so complex that you can often get plasma or serum interference.”

According to Sam Hanash of **The University of Texas M.D. Anderson Cancer Center** in Houston, it remains to be seen what technology will ultimately be used to bring these assays to the clinic. Hanash, who is studying blood-based markers for detecting early stage cancer, directs the McCombs Institute for Early Cancer Detection and Treatment.

Using fractionation and mass spec, Hanash and his colleagues have been able to find promising biomarkers at subnanogram per milliliter concentrations—concentrations too low for detection by ELISA. “The technology for research, for discovering clinical applications, are really far reaching at the present time, but we have not figured out how to achieve that level of sensitivity in a high throughput setting,” he said. “It’s clear that the discovery platform is too labor intensive and somewhat challenging to operate in a clinical setting...it doesn’t mean that the situation is hopeless.” Some promising approaches, he adds, include nanotechnology and more user-friendly mass spec. “There are a lot of other types of technology that have the potential to do the job.”

But first come the “due diligence” validation studies that must be done to determine whether or not a biomarker is indeed clinically useful, and if so in which types of patients, Hanash says. “We have to dot the i’s and cross the t’s and see where we have seen this biomarker before,” he explains. This work is done not just to reproduce the initial findings, he adds, but to find out how widely applicable a biomarker may be.

Biodesix has been spending the past five to six years doing validation work on its own test, VeriStrat, Beresford notes. “Although the discovery of algorithms and tests is important, validation is as important or more important to get those tests successfully launched and into doctors’ hands to improve decision-making,” he says. **continued »**

Proteomics

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Massachusetts General Hospital
www.massgeneral.org

Max Planck Institute of Biochemistry
www.biochem.mpg.de/en/index.html

Pacific Northwest National Laboratory
www.pnl.gov

SISCAPA Assay Technologies
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Swiss Federal Institute of Technology
www.ethz.ch/index_EN

The University of Chicago
www.uchicago.edu

University of Michigan
www.umich.edu

University of Texas M.D. Anderson Cancer Center
www.mdanderson.org

The Boulder, Colorado-based company has created a technical platform, ProTS, to analyze matrix-assisted, laser desorption ionization (MALDI) mass spectra from biological samples. VeriStrat, which is based on this technology, analyzes blood from non-small cell lung cancer patients to help physicians determine whether they should be treated with the epidermal growth factor receptor (EGFR) inhibitor erlotinib. The company is also working with several pharmaceutical companies to develop companion diagnostics.

VeriStrat can distinguish between the two-thirds of advanced non-small cell lung cancer patients who will respond well to erlotinib and the remaining third who are poor responders, according to Beresford. "We've completed numerous validation studies in lung and other cancers where VeriStrat consistently identifies patients who are likely to have different outcomes following treatment with specific therapies," he says.

'STUNNING' PROGRESS IN MASS SPEC

Some proteomics experts say mass spec will ultimately be translatable to the clinic. Preparing for this possibility, **Agilent** registered its Infinity Series 1200 liquid chromatography systems and its 6000 Series mass spectrometry systems as Class I medical devices with the FDA this January, and registered its reagent manufacturing facility with the agency last June.

While this view is not universal, consensus in general is that advances in mass spec technology have been impressive. "Over the last five years, the pace of development and application of

mass spectrometry has just been stunning," says Gilbert Omenn, a medical professor at the **University of Michigan** and chair of the **Global Human Proteome Project**.

These advances have gone hand in hand with advances in proteomics, and some leading scientists in the field are collaborating closely with companies that make mass spec machines. For example, **AB SCIEX** and Abersold are collaborating on SWATH Acquisition, a mass-spectrometry-based technique that creates complete ion maps of all the fragments and peptides in a sample by repeatedly cycling through 32 consecutive 25-Da precursor isolation windows, or swaths. Abersold and his colleagues used a fast, high-resolution quadrupole-quadrupole time-of-flight (Qq-TOF) instrument to develop SWATH, and AB SCIEX is now enabling this functionality on its TRIPLE TOF 5600 system.

Abersold compares SWATH to a satellite measuring the surface of the Earth by making several orbits and combining the information into a single image. "You could think of taking a digital photo of a crowd at a football match, and then also having photos of individuals who have been previously identified," he explains. You could then use the individual portraits to determine who was present in the group photo. "Since this all happens in the computer, we can also reexamine these maps later when new hypotheses have been generated," Abersold adds.

Richard D. Smith, director of proteome research at **Pacific Northwest National Laboratories (PNNL)** in Richland, Washington, notes that mass spec can now be used to make targeted measurements with a sensitivity matching ELISA, with assays that can be constructed very quickly. "It's becoming even faster in its rate of improvement and growth, and there are still enormous gains that are going to be coming over the next few years," adds Smith, who directs the National Institutes of Health's Research Resource for Integrative Proteomics.

Smith and his colleague Karin Rodland, chief scientist for biomedical research at PNNL, are co-principal investigators on the National Cancer Institute Clinical Proteomics Tumor Analysis Consortium, which will undertake detailed proteomic characterization of a large number of genomically well-characterized ovarian tumor samples.

The project will involve looking at how faithfully genetic changes are translated into protein levels, and investigating different protein modification states and their role. "We understand increasingly the limitations of what genomics can do and how complex the biology really is, so proteomics is essential," Smith says. For example, he adds, no biomarker at the RNA level has been found that will predict whether or not an ovarian cancer patient will respond to platinum-based drugs. But studying post-translational protein modifications may be much more revealing. "A lot of what has been done in proteomics to date has been very simplistic," he says. "We're becoming increasingly sophisticated in our ability to track posttranslational modifications of proteins."

"The project is going to really start to show in tremendous detail and depth of proteome coverage how these genomic changes play out at the proteome level," says Rodland. "We're going to learn a lot about the biology."

Anne Harding is a freelance science writer based near New York City.

DOI: 10.1126/science.opms.p1200068

New Products: Proteomics

CCD CAMERAS

The CoolSNAP MYO and the CoolSNAP KINO CCD cameras are designed to discern finer details in biological samples under lower light levels and enable scientists to achieve higher quality, higher resolution images than previous CCD technology. Packing twice as many pixels (2.8 megapixels) with a 15% improvement in peak quantum efficiency (75%), the MYO and KINO offer scientists the ability to visualize much finer details at much higher sensitivity than standard scientific CCD sensor technology. The MYO is capable of cooling to 0°C and features a fan that can be disabled, which provides flexibility to optimize cooling for longer exposures in low-light scenarios and still accommodate extremely vibration-sensitive measurements. The KINO is cooled to 20°C without a fan, making it suitable for sensitive applications such as AFM. The MYO and KINO are ideal camera technologies for immunofluorescence and fluorescent protein imaging, and also well suited for near-infrared DIC, electrophysiology, particle tracking, FRET, and FRAP imaging.

Photometrics

For info: 800-874-9789 | www.photometrics.com



IMAGE ANALYSIS SYSTEM

The PXi is a new high-resolution, multiapplication image analysis system. This powerful system is currently the best in its class for scientists who want a compact, one-click method for accurately imaging chemiluminescent and fluorescent blots as well as 1-D gels (up to 10 cm x 12 cm) stained with any type of fluorescent dye. The innovatively designed compact PXi, with its high-resolution 6.3 megapixel camera and large fixed aperture lens can quickly image even the faintest bands. It is easy to fit a range of lighting including infrared (IR) lighting and filter options inside the PXi system and using the intuitive GeneSys imaging software, the PXi can be rapidly set up to automatically select the best conditions. With just one-click, scientists can generate perfect images of chemiluminescent blots as well as 1-D gels (up to 10 cm x 12 cm) stained with any fluorescence and IR commercial dyes time after time.

Syngene

For info: +44-(0)-1223-727123 | www.syngene.com

PROTEIN PRECIPITATION SAMPLE PREPARATION

The Combipack is an economic response to the growing use of protein precipitation separation techniques in chromatography labs. Comprising four Porvair p3 protein precipitation plates and four 1 mL deep well collection plates, the Combipack provides all the elements for protein precipitation separation, at a very affordable price. Based upon the industry standard MicroLute 96-well format, the p3 plate uses the CRASH method in which proteins are denatured with acetonitrile and the flocculent is filtered out. A novel dual filter matrix retains the samples in the wells of the p3 plate until required to release by vacuum or pressure. The use of a prefilter on top of an oleophobic filter eliminates blockages commonly found with other protein precipitation plates especially when handling high protein samples. The p3 has been proven in independent tests to eliminate the mess and complex sample preparation (no centrifuging, no vortexing) traditionally associated with protein precipitation techniques.

Porvair Sciences

For info: +44-(0)-1372-824290 | www.porvairinformation.com/p3.htm

ATOMIC FORCE MICROSCOPE

The Dimension FastScan Bio Atomic Force Microscope (AFM) enables high-resolution microscopy research in biological dynamics. Breakthrough innovations in the design of the FastScan Bio system have resulted in a fast scanning AFM that allows temporal investigation under physiological operating environments in fluid while exceeding the diffraction limits of optical microscopy. The Dimension FastScan Bio system utilizes a revolutionary XYZ scanner designed to operate at high-speed rates while delivering extremely low drift and low noise, along with a small volume fluid sample cell and a user interface that removes operational complexity. Other features include an industry-proven fast scanner, automated laser and detector alignment, an easy sample engaging process, and the capability to investigate samples in a droplet of solution or its 60 µL sample cell. With the FastScan Bio, productive spatiotemporal nanometer scale research is a reality for every bio researcher.

Bruker Corporation

For info: 520-295-4373 | www.bruker.com

NITROCELLULOSE MEMBRANES

Whatman FF High Performance (HP) membranes are a new diagnostic membrane that enables fast, razor-sharp line separation and highly reproducible results for the detection of target molecules in liquids such as water, urine, blood, and saliva. The target molecules may include drugs, hormones, proteins, antibodies, nucleic acids, whole bacteria, and viruses. The FF HP membrane is suitable for use in lateral flow assay manufacture as it provides improved reliability and rapid results. The new membrane is produced using an innovative casting method to yield a uniform, new powder-free surface that delivers a coefficient of variation in capillary rise of <10%. Both the intra- and inter-lot consistency of the new membrane was tested through analysis of over 1,000 samples giving a low coefficient of variation. Three FF HP membranes are available with different capillary rise times (wicking rate); allowing researchers to choose the most suitable membrane for their assay.

GE Healthcare

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THE TRANSLATIONAL SCIENCE OF RARE DISEASES: FROM RARE TO CARE

October 8-10, 2012

Palais de Lichtenstein
Vienna, Austria

Join an international roster of academic, industrial and government scientists, headlined by Nobel Laureate Eric Kandel, who will discuss how innovative technologies are shedding light on the causes of rare diseases and strategies for developing new treatments. Topics include:

- A targeted drug for cystic fibrosis
- Exon skipping for muscular dystrophies
- Gene therapy for immunodeficiency and hemophilia
- Tailoring treatments with genomics
- Stem cells for treating blindness

For more information, including a full
list of speakers, visit dmm.aaas.org.

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POSITIONS OPEN



NEW JERSEY MEDICAL SCHOOL
UNIVERSITY HOSPITAL
CANCER CENTER
University of Medicine & Dentistry of New Jersey

INTERDISCIPLINARY TRAINING PROGRAM IN CANCER RESEARCH

POSTDOCTORAL positions are available from an NCI-sponsored Interdisciplinary Cancer Training Grant for Ph.D., M.D., or M.D.-Ph.D. recipients who seek training in basic or translational cancer research. Faculty mentors have primary appointments in both basic and clinical departments, and many are located in the newly constructed New Jersey Medical School-University Hospital Cancer Center ([website: http://njmsuhcc.umdj.edu/home/](http://njmsuhcc.umdj.edu/home/)). The Cancer Center is located within the New York Metropolitan area and contains three research floors, a 20,000 square foot mouse barrier facility, and extensive in-house Core facilities. While the principal training mechanism of this program is through mentored research with outstanding faculty, a key element of this program is a structured exposure to cancer clinical care through a unique "shadowing program." A detailed program description, list of participating faculty, and application information, can be found at [website: http://njmsuhcc.umdj.edu/home/index.php/training-program-faculty-mentors.html](http://njmsuhcc.umdj.edu/home/index.php/training-program-faculty-mentors.html). Salary support will be for two to three years for qualified applicants who must be training grant eligible (*U.S. citizen or permanent resident*). For additional information, contact **Dr. Harvey Ozer**, Program Director at **telephone: 973-972-3557**, or **e-mail ozherhl@umdj.edu**.

FACULTY POSITION in Materials Chemistry University Of California, Irvine

The Department of Chemistry at the University of California, Irvine invites applications for a tenure-track position at the ASSISTANT PROFESSOR level in the field of experimental Materials Chemistry. We are seeking a Ph.D.-level scientist who will establish a vigorous research program involving any aspect of experimental materials chemistry; a strong commitment to teach at the undergraduate and graduate levels is also required. Applications should contain a cover letter, curriculum vita, list of publications, and a description of research plans. Applicants should also arrange to have at least three letters of recommendation submitted electronically on their behalf. Completed applications should be sent electronically, via [website: https://recruit.ap.uci.edu](https://recruit.ap.uci.edu). To ensure full consideration, applications and supporting materials should be received by October 1, 2012. *The University of California, Irvine is an Equal Opportunity/Affirmative Action Employer committed to excellence through diversity and strongly encourages applications from all qualified applicants, including women and minorities. UC Irvine has an active ADVANCE Gender Equity Program.*

FACULTY POSITION in Inorganic Chemistry at The University of California, Irvine

The Department of Chemistry at the University of California, Irvine invites applications from outstanding individuals for a tenure-track position at the ASSISTANT PROFESSOR level in the broad field of Inorganic Chemistry. Candidates must have a Ph.D. in Chemistry or a related field and postdoctoral experience is desirable. The position requires both the establishment of a vigorous research program and a strong commitment to teaching at undergraduate and graduate levels. Applications must be submitted electronically via the Internet at [website: https://recruit.ap.uci.edu](https://recruit.ap.uci.edu). Applicants should upload a cover letter, a curriculum vita (including publication list), and a concise statement of research plans. At least three letters of recommendation are required. Applications and supporting materials should be received by October 15, 2012 for full consideration. *The University of California, Irvine is an Equal Opportunity/Affirmative Action Employer committed to excellence through diversity and strongly encourages applications from all qualified applicants, including women and minorities. UC Irvine has an active ADVANCE Gender Equity Program.*

POSITIONS OPEN

UIC University of Illinois
at Chicago

University of Illinois at Chicago (UIC), Department of Chemistry invites applications for a tenure-track ASSISTANT PROFESSOR in the area of materials for energy. Areas of application include energy conversion and storage, solar energy, thermoelectrics, batteries, photovoltaics, fuel cells, and catalysis. The successful candidate will be expected to carry out a full and innovative program of experimental research and to teach graduate and undergraduate courses in physical, analytical, or inorganic chemistry. Ph.D. is required. Please submit an online application (include the names and e-mail addresses of three references), and upload a cover letter, curriculum vitae, list of publications, summary of past research, and plans for future research at [website: https://jobs.uic.edu](https://jobs.uic.edu) (Click on the Job Board, then our posting) by October 1, 2012.

UIC is an Affirmative Action/Equal Opportunity Employer. Women and minority candidates are strongly encouraged to apply.

TWO ASSISTANT PROFESSORSHIPS in Evolutionary Biology University of Colorado-Boulder

The University of Colorado and the Department of Ecology and Evolutionary Biology invite applications for two tenure-track positions at the assistant professor level in evolutionary biology. The successful candidate will have an outstanding research program addressing evolutionary questions at any level of biological organization and a commitment to excellence in teaching. Applicants should assemble a cover letter, curriculum vitae, statements of research interests and teaching philosophy, and the names and addresses of three persons who are qualified to evaluate your potential for success in research and teaching. Application materials are accepted electronically at [website: https://www.jobsatcu.com](https://www.jobsatcu.com). Review of applications will begin on October 5, 2012. Contact **Dr. Andrew Martin** with questions **e-mail: andrew.martin-1@colorado.edu**.

*The University of Colorado is an Equal Opportunity Employer committed to building a diverse workforce. We encourage applications from women, racial and ethnic minorities, individuals with disabilities and veterans. Alternative formats of this ad can be provided upon request for individuals with disabilities by contacting the ADA Coordinator at **e-mail: hr-ada@colorado.edu**.*

POSTDOCTORAL POSITION The Hong Kong University of Science and Technology Division of Life Science

A postdoctoral position is available in the laboratory of **Dr. Karl Herrup**, an expert in the molecular and cellular basis of neurodegenerative disease such as Alzheimer's disease and ataxia-telangiectasia.

Projects will focus on the cell biology of neuronal cell death, but will be adjusted to fit the candidate's background and technical proficiency. The foundation of the laboratory is neurobiology, but candidates with experience in cell cycle regulation, neuroinflammation, and DNA damage are also encouraged to apply. The Herrup laboratory has recently moved from the United States to the vibrant international atmosphere of the Hong Kong University of Science & Technology, which offers an outstanding intellectual environment that is rich in technical resources. The language used in the laboratory and the University is English.

Interested individuals should send a statement of interest, a copy of their curriculum vitae, and three letters of reference to **Penny Lee**, **e-mail: bopenny@ust.hk**.

THE LAUFER CENTER FOR PHYSICAL AND QUANTITATIVE BIOLOGY AT STONY BROOK UNIVERSITY

invites applications for

Two Endowed Assistant/Associate Professors in Physical, Quantitative or Systems Biology

We seek two outstanding early- to mid-career researchers in physical, quantitative or systems biology for tenure-track positions at either the Assistant or Associate Professor levels. Research areas include, *but are not limited to*, computational, theoretical and/or experimental models of cellular networks, systems and evolution; systems pharmacology; or statistical biophysics. We seek the strongest candidates, irrespective of a particular research area within biology at the molecular or cellular levels.

Each position focuses primarily on research, with minimal teaching, and is supported by a Laufer Endowed Chair that provides annual research support. The successful individual will have considerable freedom to choose his or her departmental affiliation.

Candidates should have a PhD and a strong record of research productivity. Please apply online at <http://academicjobsonline.org>. Include a State employment application, cover letter, curriculum vitae, a two- to three-page description of research plans, and arrange to have a minimum of three letters of recommendation uploaded to the site. These positions will remain open until filled.

For more information or to obtain a State employment application, visit www.stonybrook.edu/jobs (Ref. #F-7314-12-06). Anticipated start date is September 1, 2013.

THE LAUFER CENTER is a center for research in physical and quantitative biology at Stony Brook University. In a newly renovated space on the Stony Brook campus, the Center provides a cross-disciplinary collaborative environment. The Center brings together Stony Brook researchers in chemistry, physics, applied mathematics and statistics, computer science, pharmacology, molecular genetics and microbiology, ecology and evolution; and researchers from Cold Spring Harbor Laboratory and Brookhaven National Laboratory. Stony Brook University is an exciting place for research, particularly in light of a recent major endowment, support from a New York State plan called NYSUNY 2020, and newly planned academic centers of excellence in bioinformatics, bioimaging and advanced computing.





The Molecular Biology Department at Princeton University invites applications for a faculty position at the Assistant Professor level. We are seeking an outstanding investigator to address fundamental questions in Host-Microbe Interactions.

The University has a strong commitment to interdisciplinary studies, especially in the areas of systems biology, imaging, genomics, biophysics and evolution. The department has high-level computing and microscope facilities, DNA array and high throughput sequencing technologies, mass spectrometry, and state of the art vivarium. Applicants must have an excellent record of research productivity and demonstrate the ability to develop a rigorous research program.

All applicants must have a Ph.D. or equivalent degree and a commitment to teaching at the undergraduate and graduate levels. Applications must be submitted online at <http://jobs.princeton.edu>, requisition #1200547 and should include a cover letter, curriculum vitae, a two-page research description, and contact information for three references. All materials must be submitted as PDF files. Screening of applications will begin **1 October, 2012**.

Princeton University is an Equal Opportunity Employer and complies with applicable EEO and Affirmative Action regulations.



The Molecular Biology Department at Princeton University invites applications for a tenure-track faculty position at the Assistant Professor level. We are seeking an outstanding investigator using multicellular model systems to address fundamental questions in Multiscale Cell Dynamics.

The University has a strong commitment to interdisciplinary studies, especially in the areas of systems biology, imaging, genomics, biophysics and neuroscience. The department has high-level computing and microscope facilities, DNA array and high throughput sequencing technologies, mass spectrometry, and state of the art vivarium. Applicants must have an excellent record of research productivity and demonstrate the ability to develop a rigorous research program.

All applicants must have a Ph.D. or equivalent degree and a commitment to teaching at the undergraduate and graduate levels. Applications must be submitted online at <http://jobs.princeton.edu>, requisition #1200533 and should include a cover letter, curriculum vitae, a two-page research description, and contact information for three references. All materials must be submitted as PDF files. Screening of applications will begin **1 October, 2012**.

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The Molecular Biology Department at Princeton University invites applications for a senior tenured faculty position at the Full or Associate Professor level. We are seeking an outstanding investigator to address fundamental questions in all areas of Molecular Biology.

The University has a strong commitment to interdisciplinary studies, especially in the areas of systems biology, imaging, genomics, biophysics and evolution. The department has high-level computing and microscope facilities, DNA array and high throughput sequencing technologies, mass spectrometry, and state of the art vivarium. Applicants must have an excellent record of research productivity and demonstrate the ability to develop a rigorous research program.

All applicants must have a Ph.D. or equivalent degree and a commitment to teaching at the undergraduate and graduate levels. Applications must be submitted online at <http://jobs.princeton.edu>, requisition #1200537 and should include a cover letter, curriculum vitae, a two-page research description, and contact information for three references. All materials must be submitted as PDF files. Screening of applications will begin **1 October, 2012**.

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UNIVERSITY of IOWA
CARVER COLLEGE
OF MEDICINE



Faculty Positions in Structural Biology Department of Biochemistry University of Iowa

Roy J. and Lucille A. Carver College of Medicine

The Department of Biochemistry (biochem.uiowa.edu) seeks outstanding applicants for one or more tenure track faculty positions at any rank in the area of structural biology. The department, now in the midst of a multiyear expansion, has broad research interests, and current faculty have strong collaborative interactions throughout the Carver College of Medicine and the University. Outstanding research space with state-of-the-art shared instrumentation is available. Applicants must have a relevant doctoral degree and productive research experience focusing on the application of crystallography or NMR to biomedical problems. They will be judged on their potential to initiate and maintain a vigorous, independent research program and to teach and train students and postdoctoral fellows.

To apply for this position, visit the University of Iowa website at <http://jobs.uiowa.edu>, requisition #61263. All applications should include a CV and a 3 to 5 page summary of research accomplishments and future plans. All applicants will be asked to provide names of three referees. Consideration of completed applications will begin on **October 15, 2012**.

*The University of Iowa is an
Equal Opportunity and Affirmative Action Employer.*



Harvard University | Department of Stem Cell and Regenerative Biology
hscrb.harvard.edu

Harvard University is recruiting tenure-track faculty for the multi-disciplinary **Department of Stem Cell and Regenerative Biology (HSCRB)**, Harvard's first joint Department bridging the Faculty of Arts and Sciences and the Harvard Medical School.

The **Department of Stem Cell and Regenerative Biology** is situated in the heart of the University's Cambridge campus, with research conducted in new state-of-the-art laboratories there, as well as in three of Harvard's affiliated world-class hospitals. The ultimate goal of the Department's research is combating disease and tissue degeneration and improving human health.

The Department's research and teaching mission emphasizes developmental biology, stem and progenitor cell biology, tissue and organ formation, tissue repair, regeneration, immunology, and aging. These topics are studied at the molecular, cellular, and organismic levels across a number of organ systems.

We seek to hire faculty with a history of innovative research using human, mammalian, or non-mammalian systems. We are particularly interested in applicants who are applying novel tools to advance regenerative biology and medicine. Candidates should possess an interest and aptitude in teaching undergraduate, graduate, and/or medical students and will join a dedicated core of scientists and physician-scientists utilizing stem cell and regenerative biology to inform the understanding and treatment of human disease.

Basic Scientists in the Field of Regenerative Biology

We are seeking Ph.D. scientists with demonstrated research interest in developmental and stem cell biology enabling regenerative biology – with an emphasis on organ system repair and regeneration.

Physician Scientists in the Field of Regenerative Medicine

We are seeking M.D. or M.D./Ph.D. physician-scientists with strong scientific credentials who are interested in clinical translation and who are applying novel tools to advance regenerative medicine – with an emphasis on developing new molecular and cellular therapeutics.

Application Process: Applications, including curriculum vitae, reprints of publications, statement of present and future research plans (1-3 pages), and three letters of recommendation should be addressed to Professors Paola Arlotta and Kiran Musunuru, HSCRB Search Committee, and submitted using the web-form available at <https://academicpositions.harvard.edu/postings/4194>. The submission deadline for all application materials is December 1, 2012. We strongly encourage applications from women and minority candidates.

Harvard University is an Affirmative Action/Equal Opportunity Employer.



The University of Texas Health Science Center at Tyler invites applications from outstanding scientists for state-funded faculty positions at all levels. Tyler is located midway between Dallas and Shreveport amidst the picturesque lakes, hills and forests of East Texas. The mission of the basic and clinical research at the UT Health Science Center focuses on **lung injury/repair and therapeutics, pulmonary infectious diseases, coagulation, immunology and oncology**.

Applicants in these and related fields are welcome. A **strong track record of scientific accomplishment and current extramural funding are required**. A complete listing and description of current faculty research interests can be found online via UTHSCT's website <http://www.uthct.edu/Research>. The successful candidate will bring or establish a dynamic independent research program in a discipline related to the mission of UTHSCT. Teaching in the biotechnology graduate program is encouraged but commensurate with committed research time. Our institution is growing and substantive resources are being allocated to build its translational research portfolio.

Applicants should submit their *curriculum vitae*, a statement of future research plans and the names of three references to: **Dr. Anna Kurdowska, Faculty Search Committee Chair, University of Texas Health Science Center at Tyler, 11937 US Highway 271, Tyler, Texas 75708-3154**, or by email to anna.kurdowska@uthct.edu.

 **UTHSCT** is an EEO/AA Employer M/F/V/D. This position is security sensitive and subject to Texas Education Code 51.215 which authorizes the employer to obtain criminal history information.



The University of Texas Health Science Center at Tyler invites applications from extramurally funded, outstanding scientist/administrators to serve as **Chair of Cellular and Molecular Biology**. Administrative experience required. Administrative responsibilities include oversight/development of a productive faculty group and of a biotechnology master's graduate program. Track record of successful research and current extramural funding required. Resources to support this recruitment include ample laboratory space, additional faculty positions to support growth of research programs, an endowment and unique UT System resources.

Tyler is located midway between Dallas and Shreveport amidst the picturesque lakes and piney woods of East Texas. The mission of the basic and clinical research at the UT Health Science Center focuses on **lung injury/repair, pulmonary infectious diseases, coagulation, immunology and oncology**. A new \$40 million academic building housing the Oncology Program opened in November 2011. A complete listing and description of current faculty research interests can be found online via UTHSCT's website <http://www.uthct.edu/Research>.

The successful candidate will bring or establish a dynamic independent research program in a discipline related to the mission of UTHSCT. **Preference given to programs in either lung injury and repair, aspects of matrix biology or oncology**. Associate and Full Professor level candidates will be considered. Applicants should submit their curriculum vitae, a statement of future administrative and research plans and the names of three references to: **Dr. Anna Kurdowska, Faculty Search Committee Chair, University of Texas Health Science Center at Tyler, 11937 US Highway 271, Tyler, Texas 75708-3154**, or by email to anna.kurdowska@uthct.edu.

 **UTHSCT** is an EEO/AA Employer M/F/V/D. This position is security sensitive and subject to Texas Education Code 51.215 which authorizes the employer to obtain criminal history information.

MICHIGAN STATE UNIVERSITY

Evolutionary Microbial Pathogenesis Department of Microbiology and Molecular Genetics

The Department of Microbiology and Molecular Genetics at Michigan State University seeks candidates for an open rank position at the Associate or Full Professor level in microbial pathogenesis. Applicants are sought with demonstrated expertise in molecular mechanisms of the evolution of pathogenicity, bacterial pathogenesis, microbial ecology of infectious diseases, genetics of virulence, or host-microbe interactions. Many opportunities exist for collaboration with other faculty with research interests in these areas in the Center for Microbial Pathogenesis, Center for Microbial Ecology, the BEACON/Evolution in Action Center, and the Center for Water Sciences. The person who fills this position will be expected to build and lead collaborative groups and to mentor junior faculty. A strong record of research accomplishment and an independent, externally funded research program with national visibility are required. Teaching within our graduate, professional, and/or undergraduate programs is expected. The offer will include a competitive startup package and laboratory facility.

Review of applications will begin immediately. The position will remain open until filled. Applicants must submit the following application items through the Human Resources (MAP) website at <https://jobs.msu.edu>:

- a) a letter of interest which should provide a history of funding, statement of future research and administrative plans
- b) curriculum vitae
- c) names of three potential references (not to be contacted until approval is received from the applicant)

In addition, applicants are asked to send a letter of intent only via email to the Search Committee Executive Assistant at mmgchair@msu.edu.

www.mmg.msu.edu

MSU is an Affirmative Action, Equal Opportunity Employer. MSU is committed to achieving excellence through cultural diversity. The University actively encourages applications and/or nominations of women, persons of color, veterans and persons with disabilities.

VICE CHAIR FOR RESEARCH ASSOCIATE/FULL PROFESSOR

Department of Anesthesiology

The Department of Anesthesiology and Perioperative Care at the UMDNJ-New Jersey Medical School (NJMS) seeks to recruit an established investigator for the position of Vice-Chair for Research. This position is open to outstanding MD or PhD credentialed research scientists. Evidence of a strong record of funded research and publications in perioperative medicine or pain research, including related research in neuroscience and inflammation will be given priority. We offer excellent benefits and a very competitive salary. Additional funds will be available to set up a laboratory.

NJMS is the largest medical school in the tri-state area with many nationally recognized and NIH funded biomedical research programs. There are a number of state-of-the-art core facilities and ample opportunities for collaborative research. The University Hospital, a primary teaching hospital of the New Jersey Medical School, is a widely respected tertiary care center and regional Level 1 Trauma Center. Newark is a vibrant city in the larger New York area (20 minutes from Manhattan) surrounded by the idyllic communities of Northern New Jersey. A wide variety of cultural and recreational activities are readily available.

Interested candidates should forward a CV and a letter of interest to: **Alex Bekker, MD, PhD, Professor and Chair, Department of Anesthesiology, UMDNJ-New Jersey Medical School, 185 S. Orange Avenue, MSB E-538, Newark, NJ 07103; E-mail: bekkeray@umdnj.edu**. UMDNJ is an Affirmative Action/Equal Opportunity Employer, m/f/d/v and a member of the University Health System of New Jersey.



**NEW JERSEY
MEDICAL SCHOOL**

University of Medicine & Dentistry of New Jersey



INDIANA UNIVERSITY BLOOMINGTON

Multiple Faculty Positions in Molecular, Cellular and Developmental Biology

The MCDB Program at Indiana University, Bloomington invites applications for tenure-track faculty positions at the assistant professor level. Individuals whose research complements our existing strengths in the molecular, cellular and developmental biology of eukaryotic systems are encouraged to apply. Those working on vertebrate systems and/or using genomic approaches are particularly welcome. The Indiana University Department of Biology (<http://www.bio.indiana.edu>) has over 60 research labs housed in 3 adjacent buildings. The department has state-of-the-art facilities for biological imaging, protein analysis, crystallography, genomics and bioinformatics. Applicants must hold a Ph.D. and have relevant postdoctoral experience with a strong record of research accomplishments. IU Biology faculty are expected to establish a vigorous well-funded research program and to participate in undergraduate and graduate education.

Applications received by **October 15, 2012** will be assured of full consideration. Applicants should submit a cover letter, CV, research (past, present, and planned) and teaching statement using the submissions link at <http://indiana.peopleadmin.com>. Applicants should also arrange to have three (or more) letters of recommendation sent to iuicdb@indiana.edu. Please address inquiries to **Jennifer Tarter** at 812-856-3984.

Indiana University is an Affirmative Action/Equal Opportunity Employer. Women and minority candidates are encouraged to apply.

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Ecological or Evolutionary Genomics
Assistant Professor
School of Biological Sciences,
College of Arts and Sciences

The School of Biological Sciences at Washington State University, Pullman, Washington, invites applications for a full-time, permanent, tenure-track faculty position in ecological or evolutionary genomics. This position is to be filled at the Assistant Professor level and will begin in August of 2013. Candidates should have the ability to combine cutting-edge research technologies with innovative analytics to investigate processes shaping organismal ecology and evolution. Candidates should complement our existing faculty strengths in organismal and evolutionary biology, molecular evolution, population and ecological genetics, systematics, ecology, development, and physiology. Candidates able to bring large-scale patterns of genomic, transcriptomic, or proteomic data to bear on fundamental problems in ecology and/or evolution are especially encouraged to apply.

Required qualifications include an earned doctorate at time of application, a record of research accomplishment in ecological or evolutionary genomics, evidence of commitment to teaching excellence, and effective communication skills. Successful candidates will be expected to develop and maintain an active research program supported by extramural funding, train graduate and undergraduate students, participate in graduate and undergraduate teaching, participate in service needs, and advance the university's commitment to diversity and multiculturalism.

To apply visit www.wsujobs.com and upload application materials. Applications must include a letter of application addressing qualifications, a curriculum vitae, separate teaching and research statements and three selected reprints of published or in press papers. Three (3) letters of recommendation that address the applicant's history of and potential for research, teaching and communication excellence are required. The reference letters will be automatically requested and obtained from the reference provider through our online application system. Review of applications with reference letters begins **October 1, 2012**. For information on the position or the status of your application, candidates may contact **Dr. Gary Thorgaard** (gary.thorgaard@wsu.edu). Full notice of vacancy can be viewed at <https://www.wsujobs.com>.

EEO/AA/AD

Burnett School of Biomedical Sciences
College of Medicine

Assistant/Associate/Full Professors
in Cancer, Cardiovascular and
Metabolic Diseases, and
Neurodegenerative Diseases



The University of Central Florida College of Medicine's Burnett School of Biomedical Sciences seeks outstanding scientists to establish well-funded research programs in areas relevant to Cancer, Cardiovascular and Metabolic Diseases, or Neurodegenerative diseases. Applicants with a strong emphasis on clinical collaboration and/or interests in inflammation, immune function, or physical methods of imaging (such as MRI) and diagnosis are encouraged to apply. In addition to maintaining a funded research program, active participation in undergraduate, graduate, and MD educational programs will be expected. Successful applicants must hold an earned doctorate in a discipline appropriate to the school program.

The position includes a competitive salary, startup package and laboratory space in a new 198,000 sq. ft. biomedical research building, which includes a transgenic animal facility. The approved applicant will work at the College of Medicine's new location at Lake Nona's emerging medical city. In addition to UCF's Health Sciences Campus, medical city includes the Sanford-Burnham Medical Research Institute, Nemours Children's Hospital, the Orlando VA Medical Center and the UF School of Pharmacy branch campus which provide unique opportunities for research partnerships. The Burnett School currently has 43 full-time faculty 2,463 undergraduate majors and 120 graduate students in MS and Ph.D. programs.

UCF is the nation's second largest university with over 58,000 students and is located in Orlando, a progressive metropolitan area and a major player in high-tech industry with a top ranked Research Park. Review of candidates will begin on August 1, 2012. Please apply specifying your area of interest, a curriculum vitae, a two page summary of research plans and contact information for three or more references to <http://www.jobswithucf.com:80/postings/32988>

The University of Central Florida is an equal opportunity, equal access, and affirmative action employer. As a member of the Florida State University System, all application materials and selection procedures are available for public review.



UNIVERSITÄT BASEL

The Department of Chemistry of the University of Basel has an opening for a

Senior Scientific Collaborator (100%)

in

Natural products chemistry and biology

Job description

The position will be associated to the group of Prof. Karl Gademann at the Department of Chemistry at the University of Basel and will join the research efforts in the general area of natural products chemistry. Possible topics range from the total synthesis, isolation and characterization of natural products, the genetic engineering of biosynthesis to the use of cyanobacteria with regard to sustainable energy production. The sought candidate will be responsible for the various analytical instruments in the group, in particular with regard to HPLC-MS and NMR spectroscopy. Involvement in teaching at the undergraduate and graduate level is expected, as is the organization of practical laboratory courses. The successful candidate will have the opportunity to develop a research profile by acquiring research grants and supervising research projects.

Qualifications

The applicant should have a PhD degree followed by several years of postdoctoral research in one of the areas outlined above. In addition, a very good knowledge of the German and English language is expected.

Additional benefits

We offer an exciting position at the interface of chemistry and biology at a top ranked university. The salary will be in line with the regulations of the University of Basel.

Interested candidates should direct their questions and/or send a complete resume by postal mail including list of publications and a list of 3 references (with email address) addressed to:

Prof. Dr. Karl Gademann
 Department of Chemistry
 The University of Basel
 St. Johannis-Ring 19
 CH-4056 Basel
 Tel +41 61 267 11 44 (direct) or +41 61 267 11 13
 (secretary: Mrs. Marina Mambelli)



There's only one GALILEO GALILEI

Born in 1564, Galileo Galilei once contemplated a career in the priesthood. It's perhaps fortunate for science that upon the urging of his father, he instead decided to enroll at the University of Pisa. His career in science began with medicine and from there he subsequently went on to become a philosopher, physicist, mathematician, and astronomer, for which he is perhaps best known. His astronomical observations and subsequent improvements to telescopes built his reputation as a leading scientist of his time, but also led him to probe subject matter counter to prevailing dogma. His expressed views on the Earth's movement around the sun caused him to be declared suspect of heresy, which for some time led to a ban on the reprinting of his works.

Galileo's career changed science for all of us and he was without doubt a leading light in the scientific revolution, which is perhaps why Albert Einstein called him the father of modern science.

Want to challenge the status quo and make the Earth move? At *Science* we are here to help you in your own scientific career with expert career advice, forums, job postings, and more – all for free. For your career in science, there's only one *Science*. Visit *Science* today at ScienceCareers.org.



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The Department of Chemistry and Biochemistry at The Ohio State University
seeks to fill the following tenure-track faculty positions

MATERIALS CHARACTERIZATION

Assistant Professor level to begin Autumn 2013. Candidates with research interests in state-of-the-art characterization of materials that can advance Ohio State's commitment in materials science are encouraged to apply. Demonstrated excellence in innovative research and a strong commitment to teaching are essential. Priority will be given to those candidates whose research interests complement those of existing faculty. Candidates should submit a curriculum vitae, a statement of teaching interests, and a description of a proposed research program.

Applications for this position should be posted to this position on <http://academicjobsonline.org/ajo/jobs/1702>

CHEMICAL SYNTHESIS

Assistant or associate professor level in the design and creation of small, functional molecules or materials via chemical synthesis to begin Autumn 2013. Candidates with research interests at the interface of molecular design, synthesis, and biomedical research and/or nanotechnology are encouraged to apply. Demonstrated excellence in innovative research and a strong commitment to teaching are essential. Candidates should submit curriculum vitae, publication list, and a statement of teaching and research interests.

Applications for this position should be posted to this position on <http://academicjobsonline.org/ajo/jobs/1701>.

EXPERIMENTAL BIOPHYSICAL CHEMISTRY

Assistant Professor level beginning Autumn 2013. Candidates with research interests in experimental biophysical chemistry, who develop state-of-the-art spectroscopic and/or imaging techniques and instrumentation, and apply these methods to study fundamental biochemical phenomena (for example macromolecular structure and interactions, protein folding, or energy transfer in biological systems) and can advance Ohio State's commitment in the physical chemistry of life sciences are encouraged to apply. Demonstrated excellence in innovative research and a strong commitment to teaching are essential. Candidates should submit curriculum vitae, teaching interests, and proposed research program.

Applications for this position should be posted on <http://academicjobsonline.org/ajo/jobs/1703>.

In addition, candidates should arrange to have at least three letters of recommendation submitted to the same website. For further information, contact: **Faculty Search Committee, c/o Susan Krumm, skrumm@chemistry.ohio-state.edu**. Application review begins **September 15, 2012**.

To build a diverse workforce Ohio State encourages applications from individuals with disabilities, minorities, veterans, and women. Flexible work options are available. EEO/AA Employer. Ohio State is an NSF ADVANCE Institution.



Faculty Position in Ribonomics

The Center for RNA Biology at The Ohio State University invites applications for a position at the level of tenure track Assistant or tenured Associate Professor in the broad area of ribonomics. Outstanding individuals using high-throughput laboratory and computational approaches to address important problems in prokaryotic or eukaryotic RNA biology are encouraged to apply. Information about the Center and its over 30 research groups can be found at <http://rna.osu.edu>. This appointment will be in the Department of Microbiology (<http://microbiology.osu.edu/>) and/or the Department of Molecular Genetics, <http://molgen.osu.edu/>) depending upon the research and teaching interests of the candidate.

Applications for this position should be submitted to <http://academicjobsonline.org/ajo/jobs/1740>. In addition, candidates should arrange to have at least three letters of recommendation submitted to the same website. For further information, contact: **Ribonomics Faculty Committee, at mikesell.22@osu.edu**. Candidates at the Associate Professor level may alternatively provide names of 3 references. Applications will be considered beginning **September 15, 2012**.

The Ohio State University is an Equal Opportunity Employer committed to the recruitment of candidates traditionally underrepresented on university faculties and encourages applications from women, minorities, veterans, and individuals with disabilities. Flexible work options are available. EEO/AA Employer. Ohio State is an NSF Advance Institution.



**TENURE-TRACK FACULTY POSITION IN
RNA STRUCTURAL BIOLOGY**

The Department of Chemistry and Biochemistry at The Ohio State University invites applications for tenure-track faculty position at the level of Assistant Professor in the area of RNA Structural Biology to begin Autumn 2013. Demonstrated excellence in innovative RNA research and a strong commitment to teaching are essential. The faculty member hired will also be associated with the Center for RNA Biology at OSU. Information about the Center and its over 30 research groups can be found at <http://rna.osu.edu>.

Applications for this position should be posted to this position on <http://academicjobsonline.org/ajo/jobs/1700>.

In addition, candidates should arrange to have at least three letters of recommendation submitted to the same website. For further information, contact: **RNA Structural Biology Faculty Committee, c/o Susan Krumm, skrumm@chemistry.ohio-state.edu**. Application review begins **September 15, 2012**.

The Ohio State University is an Equal Opportunity Employer committed to the recruitment of candidates traditionally underrepresented on university faculties and encourages applications from women, minorities, veterans, and individuals with disabilities. Flexible work options are available. EEO/AA Employer. Ohio State is an NSF Advance Institution.



AAAS is here – helping scientists achieve career success.

Every month, over 400,000 students and scientists visit ScienceCareers.org in search of the information, advice, and opportunities they need to take the next step in their careers.

A complete career resource, free to the public, *Science* Careers offers a suite of tools and services developed specifically for scientists. With hundreds of career development articles, webinars and downloadable booklets filled with practical advice, a community forum providing answers to career questions, and thousands of job listings in academia, government, and industry, *Science* Careers has helped countless individuals prepare themselves for successful careers.

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To learn more, visit aaas.org/plusyou/sciencecareers





Two Tenure-Track Faculty Positions in Physiology and Neurobiology

The Department of Physiology and Neurobiology in the College of Liberal Arts and Sciences at the University of Connecticut (<http://www.pnb.uconn.edu>) invites applications for TWO tenure track positions at the assistant professor level. We encourage applications from individuals who use innovative approaches to study fundamental physiological or neural processes at the molecular, cellular, or systems level. For details on the position, qualifications, and application instructions please visit <http://www.jobs.uconn.edu>. (Search #2013024)

*The University of Connecticut is an
EEO/AA Employer.*



TWO BIOCHEMISTRY FACULTY POSITIONS AT NEW MEXICO STATE UNIVERSITY

The Department of Chemistry and Biochemistry at New Mexico State University (NMSU), Las Cruces, invites candidates with a Ph.D. in Biochemistry or related area and postdoctoral experience to apply for one full-time nine-month tenure-track position at the **Assistant Professor level** (Position #199383) beginning August 2013. Expectations for this position include establishing a nationally recognized, externally funded (NIH, NSF, USDA, DOD, etc.) research program, participating in graduate and undergraduate student training, and teaching core courses at the undergraduate and graduate levels in Biochemistry. Applicants must submit a cover letter, CV, copy of transcripts, a brief description of research accomplishments and proposed research, and a statement of teaching philosophy. Go to <http://www.nmsu.edu/~personel/postings/faculty/10251147.html> for more information and application details.

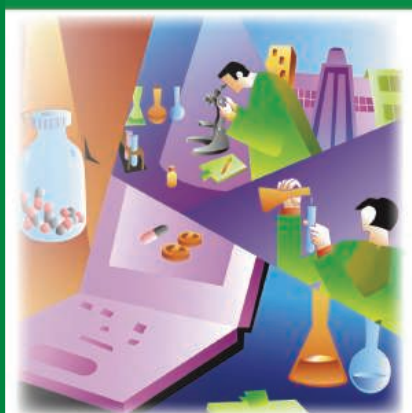
The Department also invites candidates with a Ph.D. in Biochemistry or related area to apply for one full-time nine-month nontenure-track lecturer position at the **College Assistant Professor level** (Position #197732) beginning August 2013. Primary teaching assignments will include both lower and upper division undergraduate biochemistry courses. Successful candidates must be committed to quality undergraduate and graduate education with demonstrated excellence in teaching undergraduate laboratory or lecture courses. Applicants must submit a cover letter, CV, copy of transcripts, statement of professional goals and teaching philosophy, and evidence of successful teaching. Go to <http://www.nmsu.edu/~personel/postings/faculty/1023447.html> for more information and application details.

NMSU is a public, land grant, minority-serving institution recognized by the Carnegie Foundation as a RU/H (Research University with high research activity) institution. For more information see: <http://www.chemistry.nmsu.edu/>.

Applications for either position (but not both) should be submitted via email as a single PDF file to: chembche@nmsu.edu. Reference the specific position number in the email. Candidates should arrange for three letters of recommendation to be emailed directly to: chembche@nmsu.edu. We will also accept hard copies of letters mailed to: **Biochemistry Faculty Search Committee, New Mexico State University, Department of Chemistry and Biochemistry, P.O. Box 30001, MSC 3C, 1175 N. Horseshoe Dr., Las Cruces, NM 88003-8001**. Review of Applications will begin **November 1, 2012** and continue until the position is filled.

NMSU is an Equal Opportunity/Affirmative Action Employer and encourages applications from women and underrepresented minority candidates. Offer of employment is contingent upon verification of degree and individual's eligibility for employment in the United States.

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Postdoctoral Research Fellows and Staff Scientists Center for Cancer and Immunology Research, Children's National Medical Center, Children's Research Institute, Washington, DC

Six research-track positions available to join a vibrant team of researchers in a state-of-art facility in the areas of innate and adaptive immunity (*Science* 323:1722), stem cell biology (*Cell Stem Cell* 8:399, *Sci Signaling* 2:RA75), cancer biology (*Cell* 129:1275, *Cancer Cell* 16:336; *Mol Cell* 44:770) and experimental therapy (*Nat Biotechnol* 29:428), to be advised by Prof Yang Liu and Pan Zheng. The requirements include a PhD and/or MD with <5 yrs of postdoctoral training, a solid publication record in peer-reviewed journals and skills in scientific writing. We are particularly interested in candidates with expertise in cell biology, glycobiology, biochemistry, high throughput genomics, bioinformatics, and animal models for cancer, inflammation and autoimmune diseases. The rank and compensation will be based on academic record. The academic affiliation will be with either Georgetown University or The George Washington University. We offer a competitive salary and benefits package.

To apply, please visit: www.childrensnational.org and search for requisition numbers 10367, 10368, 10369, and 10370 (research postdoctoral fellows); and requisitions 10372 and 10373 (staff scientists).

EOE, M/F/D/V



JAWAHARLAL NEHRU CENTRE FOR ADVANCED SCIENTIFIC RESEARCH Jakkur, Bangalore 560 064 INDIA

Advt. No. 11/2012 August 10, 2012

Faculty positions in Neurosciences

Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR), is a scientific institution under the aegis of the Department of Science and Technology, New Delhi. JNCASR invites applications for faculty positions at the level of Faculty Fellow/ Assistant Professor from Indian nationals working in the area of Neurosciences with an outstanding scientific record, and commitment to excellence in research and teaching. Applicants should have a PhD in biological sciences or a related discipline and minimum of three years of postdoctoral research experience.

Applications should include curriculum vitae, list of publications, summary of research accomplishments, a description of future research plans and names of three referees, and be mailed to:

Prof. Anuranjan Anand
**Jawaharlal Nehru Centre for Advanced
Scientific Research**
**Jakkur (P. O.), Bangalore 560 064,
INDIA**
Email: neuro_search@jncasr.ac.in

POSITIONS OPEN

FULL PROFESSOR AND CHAIR

Department of Biology
University of Hawai'i

The Department of Biology at the flagship Manoa campus of the University of Hawai'i seeks a senior colleague with a world-class research program and the ability to lead the department to a new level of international achievement during a major phase of growth aimed at building strength in evolutionary biology. We are particularly interested in individuals with the vision to build research strengths that will capitalize on Hawai'i's unique evolutionary legacy and position as the U.S. gateway to the Pacific Rim, as well as individuals who can foster and promote successful collaborative groups across diverse research fields (see **website:** <http://www.hawaii.edu/biology>). The department is undergoing a phase of substantial investment in human resources and will occupy a newly renovated teaching and research building in 2013. The department trains over 1,000 undergraduate majors and offers a graduate program with an enrollment of approximately 100 students. Furthermore, the department is a major contributor to an intercollege graduate degree program in Marine Biology and to a graduate specialization in Ecology, Evolution, and Conservation biology.

To apply, please send PDF formatted documents that include a vision statement for the chair's leadership role in the growth of the department, statements of teaching philosophy/experience and future research directions, curriculum vitae, three publications, and letters of recommendation from three references to **e-mail:** biochair@hawaii.edu. For a complete job announcement, please refer to **website:** <http://workatuh.hawaii.edu>. Review of applications will begin October 1, 2012 and will continue until the position is filled. Inquiries: Direct inquiries to **e-mail:** biochair@hawaii.edu or **telephone:** 808-956-8617. *The University of Hawai'i is an Equal Opportunity/Affirmative Action Institution and encourages applications from women and minority candidates.*

ASSISTANT PROFESSOR-BIOCHEMISTRY

The Institute of Molecular Biology and the Department of Chemistry at the University of Oregon (**websites:** <http://molbio.uoregon.edu> and <http://chemistry.uoregon.edu>) have an opening for a tenure-related biochemistry faculty member at the Assistant Professor level to begin in Fall 2013 or later. A Ph.D. is required and postdoctoral experience is preferred. The primary selection criteria will be the potential for establishing a vigorous independent research program addressing fundamental problems in cell and molecular biology, and excellence in teaching at the undergraduate and graduate levels. Individuals with experience using interdisciplinary approaches in areas such as biophysics, advanced imaging/structural methods, modeling, synthetic biology, systems biology, and/or chemical biology are especially encouraged to apply. We seek candidates with a demonstrated commitment to working effectively with students, faculty, and staff from diverse backgrounds. To assure full consideration, apply at **website:** <https://academicjobsonline.org/ajo/jobs/1728> by November 1, 2012. Review of application materials will continue until the position is filled. *The University of Oregon is an Equal Opportunity/Affirmative Action/ADA Institution committed to cultural diversity.*

NEON Observatory Director

The NEON Observatory Director is responsible for leading the Observatory and ensuring that the Observatory fulfills its scientific and educational mission. The Director reports to the NEON, Inc. Chief Executive Officer, but leads a multidisciplinary scientific, education, and technical staff to provide highly reliable data, infrastructure, and scientific resources to a diversity of stakeholders. The Director is also responsible for developing and managing the annual observatory budget, work plan, and reporting to the NSF. Apply at **website:** <http://www.neoninc.org>.

POSITIONS OPEN

ECOLOGIST

Assistant Professorship/Tenure Track
Department of Biology

California State University, Northridge, seeks a broadly trained field ecologist to become a tenure-track Assistant Professor of Biology. Candidates must have a Ph.D. and postdoctoral experience. The candidate's research should complement existing expertise. Teaching options include courses on ecology, statistics, and introductory biology. The successful candidate is expected to develop a vigorous research program involving undergraduate and master's students, aggressively seek extramural research funding, demonstrate teaching excellence, and provide effective instruction and mentoring to students of diverse backgrounds in a multicultural setting.

It is preferred that applicants submit their application as single PDF containing a cover letter, curriculum vitae, summary of teaching experience, statements of teaching philosophy and research interests, and three publications to **e-mail:** ecosearch@csun.edu. Applicants should also arrange to have three letters of recommendation sent to the same e-mail address.

Applicants not able to submit electronically can mail their materials to:

Ecology Search
Department of Biology
California State University, Northridge
18111 Nordhoff Street,
Northridge, CA 91330-8303

For more information **website:** <http://www.csun.edu/facultyaffairs/openings/sm/>. Screening will begin on October 19, 2012.

California State University, Northridge is an Equal Opportunity Employer committed to excellence through diversity.

ASSISTANT PROFESSOR Wildlife Ecology and Management

Purdue University invites outstanding candidates to apply for an academic-year, tenure-track faculty position at the rank of assistant professor in wildlife ecology and management. Visit **website:** <http://www.ag.purdue.edu/fmr/> for details. Ph.D. in wildlife, ecology, zoology, or related discipline and demonstrated expertise in the field of natural resources is required. The position includes research and teaching responsibilities. Submit a cover letter, curriculum vitae, summary of research interests, statement of teaching philosophy and interests, and arrange for three letters of reference to be sent to: **Search Chair, Purdue University, Department of Forestry and Natural Resources, 715 W. State Street, West Lafayette, IN 47907-2061. Telephone: 765-494-3568, e-mail:** rodw@purdue.edu. Review of applications begins 1 October 2012 and continues until the position is filled. *A background check will be required for employment in this position. Purdue University is an Equal Opportunity/Equal Access/Affirmative Action Employer fully committed to achieving a diverse workforce.*

ASSISTANT PROFESSOR-ALL AREAS Princeton University Department of Chemistry

The Department of Chemistry at Princeton University invites applications for a tenure-track Assistant Professor position in all areas of chemistry. Candidates should have a strong commitment to research and to teaching at the undergraduate and graduate levels, and are expected to have completed the Ph.D. in chemistry or a related field at the time of appointment. Applicants should submit a description of research interests, curriculum vitae, a list of publications, and contact information for three references online at **website:** <http://jobs.princeton.edu/applicants/Central?quickFind=62756>. The search committee will begin review of applications on October 17, 2012 and will continue until the position is filled.

Princeton University is an Equal Opportunity Employer and complies with applicable EEO and Affirmative Action regulations.

POSITIONS OPEN

ACADEMIC FACULTY

College of Natural Resources
University of Idaho-Moscow, ID

The College of Natural Resources (CNR) invites applications for an academic-year, tenure-track position as **ASSISTANT** or **ASSOCIATE PROFESSOR** in the field of Population Ecology/Population Dynamics. A faculty member with expertise in Wildlife Population Ecology and Modeling with strong quantitative skills is essential to research, graduate education and teaching in our programs, to CNR, to the University, and to our collaborators and constituents. The incumbent must possess a Ph.D. with a focus on population ecology and modeling with emphasis on impacts of anthropogenic and natural influences on populations of wildlife species. For a complete description and to apply online, please visit **website:** <http://apptkr.com/270067>. *Equal Opportunity Employer.*

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